

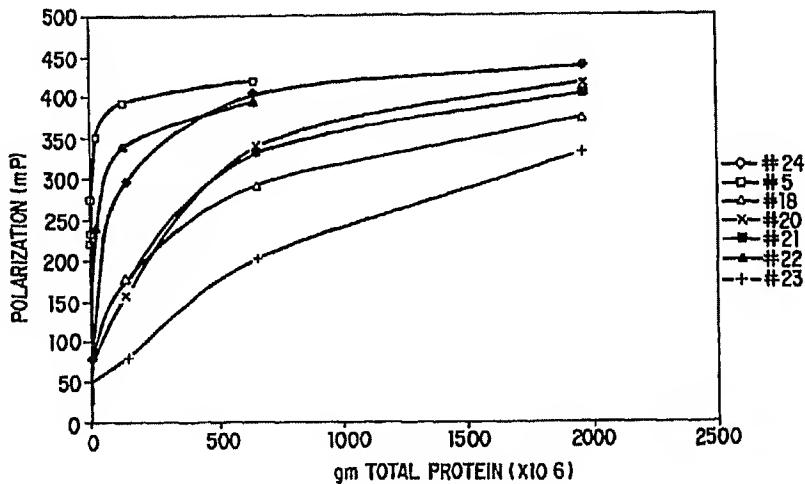
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/00, 1/68, 1/70, G01N 33/53, 33/566, 33/567, 21/00, 21/76		A1	(11) International Publication Number: WO 99/31267 (43) International Publication Date: 24 June 1999 (24.06.99)
(21) International Application Number: PCT/US98/26894			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 18 December 1998 (18.12.98)			
(30) Priority Data: 60/068,035 18 December 1997 (18.12.97) US			
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(54) Title: METHODS FOR THE SIMULTANEOUS IDENTIFICATION OF NOVEL BIOLOGICAL TARGETS AND LEAD STRUCTURES FOR DRUG DEVELOPMENT



(57) Abstract

The combinatorial screening assays and detection methods of the present invention encompass highly diversified libraries of compounds which act as fingerprints to allow for the identification of specific molecular differences existing between biological samples. The specific molecular differences identified by the combinatorial screening assay and detection methods of the present invention are potential targets for diagnosis and development therapeutics. The methods of the invention can be used in diagnostics, drug discovery, as well as genomics and proteomics. Figure (1) which is illustrative of the methods of the invention shows the interaction between molecular probes described in Table II and a human serum sample.

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**METHODS FOR THE SIMULTANEOUS IDENTIFICATION
OF NOVEL BIOLOGICAL TARGETS AND LEAD
STRUCTURES FOR DRUG DEVELOPMENT**

1. FIELD OF THE INVENTION

5 This invention relates to methods for the discovery and identification of novel biological targets of both therapeutic and diagnostic importance, and more particularly, to methods for the simultaneous discovery and identification of novel biological targets and lead structures for drug development.

10

2. BACKGROUND OF THE INVENTION

Researchers recently discovered the presence of unique chromosome material circulating in the plasma of patients having certain catastrophic illnesses (Umovitz et al.,
15 Chronic Disease Syndromes Symposium, American Society for Microbiology, May 1997). Similarly, differential expression technology was recently used to demonstrate that over 500 RNA transcripts are expressed at significantly different levels when comparing normal and neoplastic cells (Zhang. L. et al.,
20 *Gene expression profiles in normal and cancer cells*, *Science*. 276 1268-72, 1997). These discoveries illustrate the existence of the large numbers of molecular differences between normal and abnormal cells. In fact, it is reasonable
25 to assume that hundreds, even thousands of potential biological receptors exist undetected by current technology.

The technologies used to date may provide valuable information regarding targets for therapy or diagnosis, based on the identification of individual differences between
30 normal and abnormal samples. However, these technologies are not equipped to effectively provide information identifying patterns of differences observed between similar samples. Patterns of differences seen between similar samples can provide valuable information to more fully define particular
35 disease subtypes, and they may aid not only in classifying the subtypes, but also in determining appropriate therapies for a particular subtype. Clearly, with the wealth of information that is available upon the discovery of novel biological targets, and with the sheer number of such targets that remain undetected, there is an overwhelming need for efficient and effective methods for their discovery.

It has long been recognized that chemical libraries, either manmade or from natural sources, could be screened for affinity for known biological receptors such as proteins, enzymes or the like. Affinity screening can be accomplished with such known techniques as for example, fluorescence polarization, scintillation proximity assays, enzyme-linked immunosorbent assays, and the like. Recently, a porous silicon biosensor capable of detecting virtually any molecule that binds with high affinity to another molecule has also been disclosed. When the biological receptor is a target of therapeutic importance, library members that exhibit high affinity and specificity for the target can be of value in diagnosis and/or drug development. The advance of combinatorial chemistry has vastly increased the numbers of ligands available for affinity screening.

Scintillation proximity assays (SPA), for example as described in U.S. Patent 4,568,649, are used to test vast chemical libraries for affinity for a known receptor. In a standard SPA assay, the receptor is tagged with a scintillant-loaded bead and screened against radiolabelled ligands in solution. If the labeled ligand has affinity for the receptor and becomes bound, the resulting proximity of the radiolabelled ligand and the scintillant in the beads leads to activation of the scintillant and the emission of light. If the labeled ligand has little or no affinity for the receptor, the radiolabel will not accumulate sufficiently close to the scintillant to allow for energy transfer from radioactive decay and little light emission will be detected. One significant drawback to SPA is the presence of "noise" or background radioactivity in the system caused by the nonspecific adsorption of labeled ligands. For this reason, the beads are typically incubated with a blocking agent such as albumen, detergent or powdered milk to block sites responsible for such nonspecific adsorption. Another significant problem with SPA is that the requirement of using radioactive substances, poses a health hazard, such substances are difficult to dispose of, and are expensive to use.

A modification of the SPA assay has been used in competitive-type screening procedures where the receptor is immobilized to a scintillant-loaded bead and then placed in a solution containing a radiolabelled substrate for that receptor. Ligand samples are then added to the mixture and

any compound that successfully competes with the substrate for the immobilized receptor will reduce the amount of emitted light. The use of SPA in a high throughput screen is described in Wang, P., *Target Identification, Assay Development and High Throughput Screening in Drug Discovery, 5 in Sino-American Pharmaceutical Professionals Association (SAPA), The 5th Regional Symposium on Drug Discovery and Development, 1997*, Kenilworth, NJ.

Fluorescence polarization is another frequently used 10 assay system for identifying compounds that have affinity for a particular receptor. When a fluorescent molecule is attached to one end of an oligomer connected to a ligand, the binding of a receptor to the ligand severely limits the rotation of the fluorescent molecule. When polarized is 15 passed through a solution containing the fluorophore-tagged oligomer having a receptor bound or absorbed thereto the light that is emitted is also polarized.

Another affinity screening technique was recently disclosed by Lin, V., Motesharei, K., Dancil, K., Sailor, M., 20 and Ghadiri, M. in *A porous silicon-based optical interferometric biosensor*, Science 278, 840-43 (1997). In essence, this technique involves the use of a silicon wafer that has been etched to create a porous surface. When light is shone on the porous material, an interference pattern is 25 created whose position shifts when the refractive index of the surrounding medium changes. Using well-known chemistry, various molecular recognition elements are attached to the porous surface which is then reacted with a source of target molecules. When the probing element tied to the porous 30 surface binds to a target molecule, the resulting change in refractive index causes a shift in the interference pattern that can be detected by a charge-coupled device detector. The biosensor can detect tiny (e.g., femtomolar) concentrations of DNA sequences as well as recognize small 35 organic molecules. The recognition elements of the sensor may be based upon virtually any supramolecular interaction such as, for example, nucleotide hybridization, enzyme-substrate binding, lectin-carbohydrate interactions, antibody-antigen binding, host-guest complexation, and the like.

Combinatorial chemistry is capable of creating libraries containing hundreds of thousands of compounds, many of which

may be structurally similar. While high throughput screening programs are capable of screening these vast libraries for affinity for known targets, new approaches have been developed that achieve libraries of smaller dimension but which provide maximum chemical diversity. (See, e.g., Matter.

5 *H. Selecting Optimally Diverse Compounds from Structure Databases: A Validation Study of Two-Dimensional and Three-Dimensional Molecular Descriptors*, Journal of Medicinal Chemistry, 1997, 40:1219- 1229).

10 Affinity fingerprinting has previously been used to test a discrete library of small molecules for binding affinities for a defined panel of proteins. The fingerprints obtained by the screen are used to predict the affinity of the individual library members for other proteins or receptors of interest. The fingerprints are compared with fingerprints obtained from other compounds known to react with the protein of interest to predict whether the library compound might similarly react. For example, rather than testing every ligand in a large library for interaction with a known 15 receptor that is associated with a particular pharmacological activity (for example, antihistaminic or anticholinergic activity), only those ligands having a fingerprint similar to other compounds known to have that activity would be tested. (See, e.g., Kauvar, L.M. et al., *Predicting ligand binding to 20 proteins by affinity fingerprinting*, Chemistry and Biology, 1995, 2:107-1 18; Kauvar. L.M., *Affinity fingerprinting*, Pharmaceutical Manufacturing International. 1995 8:25-28; and Kauvar, L.M., *Toxic-Chemical Detection by Pattern Recognition 25 in New Frontiers in Agrochemical Immunoassay*, D. Kurtz. L. Stanker and J.H. Skerritt. Editors, 1995, AOAC:Washington, D.C., 305-312).

30 While the technologies and assays used to date are of value regarding known targets or receptors for therapy or 35 diagnosis, they are very specific in nature and thus, provide limited information. Differential expression, for instance, focuses only on the detection of different levels of mRNA expression, it does not detect the existence of sequence differences. Moreover, this approach provides no information regarding other changes in the biological system such as post-translational modifications, that do not result in altered expression levels, nor in most cases does it provide

information regarding the response of neighboring cells to abnormal cells.

Thus, there exists a need for a detection method which is able to broadly detect specific differences and changes between biological samples. Such a method will also provide 5 a method that facilitates the identification of biological targets and lead structures for diagnostic and drug development.

3. SUMMARY OF THE INVENTION

10 The combinatorial screening assays and detection methods of the present invention utilize highly diversified libraries of compounds to interrogate and characterize complex mixtures in order to identify specific molecular differences existing between biological samples, which may serve as targets for 15 diagnosis or development of therapeutics.

The invention is based, in part, on the Applicant's design of sensitive, rapid, homogeneous assay systems that permit the evaluation, interrogation, and characterization of samples using complex, highly diversified libraries of 20 molecular probes. The ability to run the high throughput assays in a homogeneous format increases sensitivity of screening. In addition, the homogeneous format allows the molecules which interact to maintain their native or active conformations. Moreover, the homogeneous assay systems of 25 the invention utilize robust detection systems that do not require separation steps for detection of reaction products. In a preferred embodiment, fluorescence polarization is used to detect interactions between the sample and library components.

30 In an alternative embodiment, the assay can be run in a heterogeneous format in which one of the reaction components (e.g., either the library or the sample components) is releasably linked to a solid support. The reaction products resulting from contacting the sample with the library are 35 released into the liquid phase, and can advantageously be detected using robust detection systems, such as fluorescence polarization.

The assays of the invention can be used for diagnostics, drug screening and discovery, target-driven drug discovery, and in the field of proteomics and genomics for the identification of disease markers and drug targets.

In one embodiment, the highly sensitive combinatorial screening assays and detection methods of the invention utilize ligand/receptor interactions as a discovery tool to detect receptors that exist in complex biological mixtures but that may have been hitherto undetected. The 5 combinatorial screening assays and detection methods of the present invention evaluate the binding interactions of a diversified universe of ligands with the potentially thousands of receptors in a biological sample and identify binding interactions that are unique to or characteristic of 10 the sample, which may be indicative of a certain pathology (including, but not limited to, disease, disorder and infection). Thus, the present invention identifies not only novel receptors, but also characterizes specific differences occurring between normal and diseased cells and/or tissues of 15 the same type. The screening assays and detection methods of the present invention provide much more information than existing target discovery methods such as genomics or differential expression techniques that focus on detecting genotypic changes and detect only DNA or mRNA changes; 20 moreover, proteomics do not detect non-protein ligands, and therefore only relate to proteins encoded for by the particular sequences. By contrast, the methods of the present invention detect all types of ligand/receptor interactions, whereby the receptor could be proteins, 25 carbohydrates, nucleic acids, or any molecules with a shape that is capable of binding interactions.

In accordance with the methods of the present invention, it is possible to evaluate the binding affinities of a library of diverse molecules for the thousands of potential 30 binding sites present in a complex biological sample and generate a pattern of binding affinities exhibited by the sample which provides a unique fingerprint for that sample.

The present invention provides methods for "fingerprinting" complex biological mixtures, such as for 35 example, blood serum, using diverse libraries of chemical compounds. The specific binding interactions that result between the members of the library and molecules in the biological sample provide a unique fingerprint for the sample. Such fingerprinting allows for the identification of novel interactions occurring in a particular sample, and when the sample involves a particular pathology, the fingerprint allows for the identification of specific phenotypical

differences existing between normal and abnormal, or diseased and undiseased cells and/or tissues of the same type.

Furthermore, once novel interactions have been identified, the molecules involved may be used as lead structures, receptors or targets for diagnostic and/or pharmaceutical

5 development.

The screening assays of the present invention encompass not only the identification of individual novel receptors, but even more importantly, identify a pattern of binding interactions that is characteristic of a particular pathology 10 or class of pathologies. Such patterns of binding affinities provide valuable information useful in more fully and precisely defining and classifying pathology subtypes. Precise diagnostics are important not only to the detection of disease, but also to the management and therapy of 15 disease, all of which in turn allow for predictable clinical outcomes.

Utilizing the principles of the present invention, samples from many different individuals may be collected and tested for interactions with a defined library, and the 20 resulting data used to create a database incorporating all of the binding interactions identified. Data relating to samples obtained from individuals exhibiting a pathology or disease of interest may then be extracted for analysis and compared with the remaining records in the database to 25 identify interactions or patterns of interactions that would be predictive of the pathology or disease state. Interactions and patterns so identified can be used not only to characterize and classify the particular pathology or disease state, but can also be used to format a diagnostic 30 test or to develop a therapy or pharmaceutical.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-5 are graphic representations of the data compiled in Examples 4 through 7, respectively.

35 Figure 6. The Differential Binding of Probes to Human and Bovine sera. The graph depicts the fluorescence polarization values obtained for human and bovine sera using various probes.

Figure 7. The Differential Binding of Probe 18 to the Biological Samples Listed in Table 7. The fluorescence polarization values for each sample using Probe 18 are depicted graphically.

Figure 8. The Differential Binding of Probe 70 to the Biological Samples Listed in Table 7. The fluorescence polarization values for each sample using Probe 70 are depicted graphically.

Figure 9. The Structure and the Differential Binding 5 Pattern of Probe 147. (A) The structure of Probe 147 is depicted. (B) The graph depicts the fluorescence polarization values obtained for each sample listed in Table 7 using Probe 147.

Figure 10. The Structure and the Differential Binding 10 Pattern of Probe 129. (A) The structure of Probe 129 is depicted. (B) The graph depicts the fluorescence polarization values obtained for each sample listed in Table 7 using Probe 129.

Figure 11. The Structure and the Differential Binding 15 Pattern of Probe 135. (A) The structure of Probe 135 is depicted. (B) The graph depicts the fluorescence polarization values obtained for each sample listed in Table 7 using Probe 135.

Figure 12. Differential Binding of Probe D6. The 20 fluorescence polarization values for culture samples of *Staphylococcus aureus* (SA), methicillin resistant *Staphylococcus aureus* (MRSA), *E. coli*, and uninoculated Brain Heart Infusion medium (BHI) using fluorescein labeled probe 25 D6 were determined. The graph depicts the fluorescence polarization values obtained for each bacterial strain and uninoculated BHI medium after various culture incubation periods.

Figure 13. Classification of the Bacterial Cultures 30 Based Upon the Fluorescence Polarization Values Obtained Using Probe D6. The results from the fluorescence polarization assays for the twelve unknown cultures were grouped based upon their fluorescence polarization values.

Figure 14. The Differential Binding of Probes to Normal 35 (Lot HS300) and Diabetic Human Sera. The graph depicts the fluorescence polarization values obtained for normal and diabetic human sera using a variety of probes.

Figure 15. The Differential Binding of Probes to Lipid Depleted Diabetic and Normal (Lot HS300) Human Sera. The graph depicts the fluorescence polarization values obtained for lipid depleted normal and diabetic sera with various probes.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of a compound that binds with high affinity to a target molecule as not only a possible lead compound for pharmaceutical development, but also its use as an informational molecule.

5 The frequency with which a compound in a diverse combinatorial library of chemicals strongly binds to a particular target is rather low (perhaps 0.0001 percent or less), but if there are a large number of targets this frequency increases dramatically. For example, if instead of
10 a single target molecule there are 1000 possible target molecules the probability of obtaining a "hit" increases to 0.1 percent. Complex biological systems contain thousands of different molecules many of which may be altered in a particular disease state; for example, prostate or breast
15 cancer. The probability, therefore, of obtaining high affinity interactions between members of a diverse, combinatorial library of organic molecules and informative components of a clinical sample are much higher than the probability of obtaining a "hit" for a single target. When
20 these interactions are detected and quantitated in accordance with the present invention a rather complete picture of the sample is formed (depending upon the number and diversity of the library components of the system being evaluated).

The combinatorial screening assays and detection methods
25 of the present invention encompass highly diversified libraries of compounds which act as fingerprints to allow for the identification of specific molecular differences existing between biological samples. The specific molecular differences identified by the combinatorial screening assay
30 and detection methods of the present invention are potential targets for diagnosis and development of therapeutics. The successful application of the combinatorial screening assays and detection methods of the present invention requires at least three components: (1) a diverse molecular library
35 (probe(s)); (2) a source of clinical samples (control and test sample); and (3) a sensitive assay for detecting interactions of members of the library with components of the sample.

Accordingly, one aspect of the present invention is a method for characterizing a pathology, said method comprising identifying a pattern of binding interactions between (a) receptors or targets present in biological samples

associated with the pathology, and (b) a library of ligands or probes, wherein the pattern of binding interactions provides a unique fingerprint for the pathology. In accordance with the present invention, a "receptor" or "target" is a biological molecule which demonstrates a binding affinity or interaction with a ligand or probe. Examples of such receptors or targets include any biologically active molecule that is differentially expressed or modified and is capable of interacting with a probe or ligand, but are not limited to, proteins including enzymes, lipids, nucleic acids, including DNA, RNA, carbohydrates, antigens, antibodies, etc. Examples of ligands or probes include any molecule, either natural or synthetic, which may be used to interact or bind to the receptors or targets to measure or indicate the presence of said ligand or probe in a given biological sample.

In a particular embodiment, the combinatorial screening assays and detection methods of the present invention may be used for the detection or characterization of a pathology. In accordance with this embodiment, the pathology to be identified or characterized may include, but is not limited to, a transformed phenotype, genetic defect, malignancy, cancer, tumor, genetic disorder, viral infection, bacterial infection, fungal infection or the presence of a parasite.

Vertebrates, particularly human beings, are physically complex. It is this very complexity that has made accurate diagnostics difficult to develop and time consuming to perform. For example, blood plasma contains thousands of proteins, carbohydrates, lipids and nucleic acids. Changes or alterations in any one or many of these components may be highly diagnostic of a particular disease state or of the outcome of a particular therapy and yet the very complexity of the system prevents accurate detection and analysis of these changes. In one aspect, the present invention allows for the rapid analysis of very complex biological systems such as blood plasma or sera, tissue homogenate, cerebrospinal fluid, urine, sputum or any other clinical material, including but not limited to, those materials that can be obtained or prepared in a fluid form.

One embodiment of the present invention is medical diagnostics. Areas of diagnostics where the present invention is applicable include, but are not limited to the

early detection of various types of cancer; the detection and identification of infections; the detection of toxic side effects of therapeutics; and other disease states and pathologies. With regard to any given pathology, numerous physiological changes occur within an organism's complex

5 biological system. Some changes are a direct result of the pathology while others result from the body's response to the pathology. Any or all of the changes may be diagnostic. Unfortunately, for most diseases the diagnostic changes are unknown and prior to the present invention there was no

10 sensitive, rapid method for detecting these diagnostic changes. In some cases antibody based diagnostics are used to detect a novel antigen associated with a particular pathology (for example, the PSA antigen associated with prostate cancer), but antibody based diagnostics depend on

15 first identifying the antigen. Also, antibody production is both expensive and time consuming. In the present invention the binding interactions of large, diverse libraries of chemicals to biological samples, blood plasma, for example, from individuals with a particular pathology are compared

20 with samples from a normal population and the differences are analyzed. The differences may be qualitative or quantitative, and may result from binding to any molecule, such as, for example, proteins, lipids, carbohydrates or nucleic acids, enzymes, antigens, or the like. The method is

25 not biased as to the type of molecule it can detect. The binding interactions may result in the discovery of a single or a small number of binding interactions that are diagnostic for the pathology under study, or alternatively, a unique pattern may be recognizable. Either way the identified

30 unique binding interactions can be assembled into an inexpensive, rapid fluorescence polarization based assay that can be done in the physician's office or at bedside.

One application for the present invention is the diagnosis and treatment of various types of cancer, such as,

35 for example, prostate, cervical and ovarian cancers. Currently, prostate cancer diagnosis relies upon an antibody-based detection of the PSA antigen. Recent evidence from others indicate that elevation of the level of certain cytokines may also be diagnostic for this disease. These two indicators are probably not some of the earliest indicators of prostate cancer that are actually present and the present invention provides a rapid method for the identification of

other yet unknown diagnostic indicators. Accordingly, the present invention enables the discovery of new diagnostic markers by probing, for example, blood plasma samples from individuals with prostate cancer as well as control populations with a library of fluorescent probes. The

5 discoveries made using this screening assay may then result in the development of therapeutics. Thus, the present invention includes the discovery of probes or markers for use in diagnostic assays or kits, and their subsequent use in drug discovery.

10 The present invention may also be useful for the detection of diagnostic markers in Pap smear samples. In accordance with the present invention, normal and abnormal Pap smear samples obtained from individuals can be screened with a library of labeled probes and those binding

15 interactions that are diagnostic of malignancies or infectious disease can be used in fluorescence polarization-based diagnostics. Furthermore, the discovery of biological molecules associated with a malignancy or infectious disease can then be used to develop therapeutics.

20 The present invention can be used to screen for a biomarker(s) for ovarian cancer, which can be used to develop a diagnostic kit for ovarian cancer. Recently, ovarian cancer activating factor (OCAF) has been suggested to serve as a biomarker for ovarian cancer. Currently, a serum assay

25 for CH125, a biomarker for ovarian cancer, is used to detect ovarian cancer. In accordance with the present invention, the fluorescence polarization assay, the DNA obstruction assay, and the scintillation proximity assay (SPA) described in the present invention can be used in clinical studies to study

30 known biomarkers for ovarian cancer (OCAF and CA125) and to identify new biomarkers for ovarian cancer. These assay systems will provide simple, rapid, sensitive, and accurate means of screening clinical samples with a labeled probe or libraries of labeled probes.

35 For example, the fluorescence polarization values for the interaction of fluorescent compounds with specimens (blood, serum, plasma or other bodily fluids) obtained from patients with ovarian cancer can be compared with the fluorescence polarization values obtained for clinical samples from patients with other types of gynecologic cancer, non-gynecologic cancers, and no cancer. An altered polarization value for specimens from patients with ovarian

cancer as compared to the other samples will indicate that an agent binds differentially to a component(s) present in the specimen from ovarian cancer patients. Thus, this agent may be a useful biomarker to distinguish ovarian cancer specimens from non-ovarian cancer specimens. A fluorescence

5 polarization value for specimens from patients with ovarian cancer that does not differ significantly from other samples will suggest that the agent is not useful as a biomarker to distinguish ovarian cancer specimens from non-ovarian specimens. Those agents identified that differentially bind

10 to different types of cancers can be used alone or in combination with other agents to detect and diagnose different types of cancers. Moreover, the identified agents can be used to develop therapeutics for the treatment of cancer.

15 The present invention also provides methods for identifying an early indicator of the onset of diabetes. Current methods for the detection of diabetes are useful only after the disease is fully developed and much damages has already been done. Indeed, diagnosis is done only after

20 gross symptoms are present. In other words, current diagnostic procedures merely confirm that the disease is present. The methods described in the present invention provide methods for identifying agents that are early indicators of diabetes. For example, the glycosylation of

25 blood proteins, a characteristic of diabetes, creates unique binding sites that can be detected with probes from a tagged library of diverse molecules using the methods described in the present invention. Those probes that are identified to be early indicators of the onset of diabetes can be used in

30 accordance with the invention in a fluorescence polarization-based assay. This assay can be used as a diagnostic in the physician's office or at a diagnostic laboratory.

The present invention further provides methods for identifying and distinguishing infectious microorganisms, as

35 well as the diseases that they cause. Infectious microorganisms produce a wide range of mostly uncharacterized proteins, carbohydrates, and other molecules when present in their host. Also, in response to infection the host organism produces unique proteins, such as, for example, specific antibodies, specific receptors, chemokines, and cytokines. All of these unique molecules are candidates for binding to probes and therefore, represent new diagnostic targets for

infectious diseases. The present invention provides methods for identifying binding candidates that differentiate between infectious microorganisms and/or the diseases that they cause. The binding candidates identified can then be used for diagnosing infectious diseases and may lead to the 5 development of a pharmaceutical therapy. Example 10 describes the discovery of a unique probe from a small library of probes that allows *Staphylococcus aureus* to be distinguished from methicillin resistant *Staphylococcus aureus*.
10

The present invention is not only applicable to the detection of infectious bacteria, but also other infectious agents, such as, for example, fungi, parasites, viruses and perhaps, even prions. For example, neuraminidase inhibitors 15 have recently been developed that inhibit the Influenza A and B viruses. The effective utilization of these drugs requires that a rapid diagnostic kit be available to identify the presence of the influenza virus. The present invention provides methods for discovering tagged molecules with high 20 avidity for viral neuraminidase or some other viral component. These probes can then be incorporated into a rapid diagnostic test for the presence of the influenza virus.

The above discussion demonstrates certain of the 25 benefits of using the assay systems described in the present invention to test known agents and to identify new agents for their ability to differentially bind to clinical samples. Those agents identified can be used alone or in combination with other agents to detect and diagnose pathologies or 30 diseases. Once identified using the methods of the present invention, such agents can be used as diagnostics (e.g., within diagnostic kits) that can be used rapidly and inexpensively to detect and diagnose various pathologies or diseases.

35 Another aspect of the present invention is a method for identifying biological targets of therapeutic and diagnostic importance and lead structures for drug development, comprising contacting two biological samples with two identical libraries of probes, wherein one biological sample is a control; detecting the binding interaction between each probe and a component of the biological samples; and identifying binding interactions that are characteristic of

the second (non-control) biological sample. The ligand or target so identified is a biological target and the probe having affinity for the biological component is a lead structure for drug development.

In a preferred embodiment, the second biological sample 5 comprises serum from a diseased individual or diseased or otherwise abnormal cells or tissues of the same type as the control. As diagnostics are typically based upon the presence of a detectable biological target, the method is therefore of value in the development of diagnostic tools 10 related to the pathology associated with the cells or tissues being screened. Moreover, because each time a novel receptor or target is discovered, a compound having affinity for the receptor is also discovered, the method is a source of lead structures for the development of a pharmaceutical for use in 15 the treatment of the pathology.

In still another embodiment, the principles of the present invention may be used for the toxicological screening of potential pharmaceuticals. The invention includes methods for determining the toxic properties of a compound as well as 20 methods for determining the presence of toxic compounds in an individual by measuring the biological response to toxic compounds. Many drugs as well as their metabolites have adverse side effects or are toxic. There is a very strong need for earlier indicators of toxicity that are predictive 25 of damage rather than merely confirmatory that damage has occurred. At present, the prediction of toxic properties, or side effects, is difficult during the early stages of drug development that occur prior to clinical testing. By utilizing the principles of the present invention, the 30 presence or absence of toxic properties may be predicted at a much earlier stage in development than is currently possible. Initial studies to detect these early warning signals can be accomplished with cell culture studies utilizing liver cells, for example. In this approach, liver cell cultures can be 35 treated with known toxins and, over time, samples taken and probed with a diverse library of tagged compounds. Binding interactions that occur in the treated cultures, but not in control may be early markers for cell or tissue damage. These probes can then be tested in animal model systems. Thus, the present invention offers methods of detecting early markers that are predictive of impending damages due to drugs of other chemicals.

In accordance with this aspect of the invention, cultured cells or tissues are treated with known toxic or non-toxic compounds, and then extracts are prepared from the cultures and probed with a library of labeled compounds. This probing with the library of labeled compounds provides a 5 "fingerprint" which reflects the responses of the cells or tissues to the toxic or non-toxic compounds. Any changes in the fingerprint patterns correlated with toxic properties are analyzed, and optionally further classified. Subsequently, cultured cells or tissues for which a fingerprint pattern has 10 been established as set forth above are treated with a test compound having unknown toxic properties, and extracts are prepared from the treated cultures and probed with the library of labeled compounds. The resulting fingerprint or profile of the cell's or tissue's responses is then compared 15 with the reference profiles generated for that cell or tissue using the known toxic/non-toxic compounds to predict the nature of the test compound.

In still another embodiment, the principles of the present invention may be used to characterize the function of 20 an expressed protein. Genome sequencing projects, including The Human Genome Project, create large databases of gene sequences, which can be cloned and the proteins encoded by these genes can be expressed. Unfortunately, knowing the sequence of a gene or expressing a protein from that sequence 25 often does not reveal the function of that protein, its role in disease, or other molecules the expressed protein interacts with in the living cell.

Recognizing the shortcomings of genomics in providing this information, several companies have adopted the 30 "Proteomics" approach. In Proteomics, two-dimensional (2-D) Polyacrylamide Gel Electrophoreses (PAGE) is used to separate proteins according to charge and mass. The resulting protein patterns are then compared and attempts are made to stratify patient populations according to unique patterns. This 35 approach has the advantage of dealing with Phenotype (what is) as opposed to Genotype (what may be). It is well known that 2-D electrophoresis fails to detect or resolve a large number of proteins in very complex samples. The methods of the present invention do not have this problem because binding of a tagged probe to small, medium or large targets gives a clear discernible signal. Furthermore, the present invention is three-dimensional (3-D) and except where the

target molecule is very small (less than a few thousand daltons) a binding event gives a clearly discernable signal. Also PAGE is designed to detect proteins whereas the present invention is designed to detect a binding interaction between a labeled probe and any biomolecule larger than a few
5 thousand daltons. Finally, running and analyzing 2-D gels is time consuming, labor intensive and very expensive. The methods of the present invention are on the other hand, rapid and very inexpensive. In any direct comparisons, the methods of the present invention to outcomes predictions is
10 preferable to either Proteomics or Genomics.

The present invention provides a means to clarify the biological function of an expressed protein. When expressed proteins are probed with tagged libraries of small organic molecules some number of probes can be found that bind to the
15 binding sites of these proteins. These probes themselves are potential inhibitors of these expressed proteins. These probes can be added to cell culture or other systems to determine their biological effects and thus be used to define the activity of this protein. Alternatively, the tagged
20 probes can serve as an artificial substrate for competitive assays with unlabeled molecules in order to discover inhibitors of even stronger avidity. These unlabeled molecules can then be tested in model systems to determine the effect of these agonists or antagonists on cellular
25 function. In this manner, the function of a previously unknown or uncharacterized expressed protein can be determined using the technology described by the present invention.

Genes from sequencing genomes of organisms from bacteria
30 to humans are now being cloned and expressed on a large scale. The present invention provide methods for rapidly unraveling the function of these proteins and at the same time discovering leads for developing new pharmaceuticals. In addition, the invention includes methods for discovering
35 potential inhibitors of, or affinity ligands useful for the purification or analysis of proteins for which no enzymatic or structural function has yet been ascribed. Such protein targets include those identified by genomic approaches.

The present invention also provides methods, which can be utilized to stratify populations for any purpose. For example, this technology may be used to stratify groups for clinical trials. Moreover, the present invention allows mass

screening for the early detection of pathologies or for analysis of the distribution patterns of various disease inexpensively. Once informative probes have been developed, the cost of screening is very low. Screening requires only an inexpensive fluorescence polarization instrument and the 5 probes. The probes are not only inexpensive to manufacture, but only nanomoles are required for an assay.

Numerous other aspects, features and advantages of the present invention will become readily apparent from the following detailed description of the preferred embodiments 10 and the appended claims. It should, however, be understood that the present disclosure is to be considered as an exemplification of the principles of the invention and is not intended that the invention be limited by the aspects herein described.

15

5.1 DIVERSE LIGAND LIBRARIES OF PROBES AND DETECTIONS THEREOF

In accordance with one embodiment of the present invention, "fingerprints" are established when biological 20 samples, such as blood, sera, body fluids, such as urine, cerebrospinal fluid, amniotic fluid, saliva, mucous, tissue samples, cells, viruses, microorganisms, or organic molecules including RNA, DNA, peptide and proteins, and small organic molecules are exposed to a battery of known reagents to 25 generate a panel of values which reflect a pattern of binding interactions. In accordance with the present invention, the diverse ligand library comprises the battery of known reagents or probes, which interact or bind to receptors or targets, to measure or indicate the presence of the receptors 30 or targets in a given biological sample. The ligands or probes of the present invention include, but are limited to, any biological molecule, either natural or synthetic, and may include, but are not limited to, nucleic acids, including DNA or RNA, small organic molecules, peptides, glycoproteins, 35 proteins, polysaccharides, saccharides or inorganic molecules.

In accordance with the present invention, ligand or probe libraries may be used to generate differential binding patterns to accurately distinguish between biological samples. In a preferred embodiment of the present invention, diverse ligand libraries encompass known molecules which when exposed to a biological sample generate a panel of values

which reflect a pattern of binding interactions. When the fingerprints of the present invention are used to distinguish between samples, for example to identify or distinguish a particular microorganism, such as a particular strain of virus, bacteria, parasite or fungus, the diverse library of

5 ligands should include ligands or probes known to specifically or non-specifically interact with a component of the microorganism.

In yet another embodiment of the present invention, when the receptor or target is unknown, the biological sample to be tested may be exposed to a library of ligands or probes composed of molecules which are in part, randomly selected, thus allowing for the identification of unique binding interactions which may then be identified by known methods.

A receptor that is found to exist in only the test sample, or 15 in a different concentration in the test sample, may serve as a potential target for diagnosis or treatment of the particular pathology associated with the test sample. In addition, the ligand found to have high affinity and specificity for the receptor provides a lead structure for 20 drug development.

Upon contacting a biological sample with a characterized supply of ligands, a ligand having affinity for a receptor existing within the sample will bind to the molecule, forming a ligand/receptor conjugate that can be identified using 25 various assay techniques. Ligand/receptor interactions occurring in a normal or control sample can be compared with those occurring in a second sample that is comprised of abnormal cells or tissues of the same type as the control to identify specific differences between the two. The 30 probes/ligands that are used in the assay systems described can be labeled, tagged, or conjugated such that a detectable signal is generated when a component of a biological sample binds to a probe. The probes/ligands may be labeled with labels known in the art, including but not limited to 35 radioisotopes, fluorescent molecules, chemiluminescent compounds, and bioluminescent compounds.

For use in the scintillation proximity assay, the molecules are preferably labeled with a radioisotope, including but not limited to ^{32}P , ^{35}S , ^{125}I , or ^{131}I . The radioactive isotope can be detected by a gamma counter or a scintillation counter.

Probes/ligands may also be labeled with a fluorescent molecule such as fluorescein (FL), rhodamines, 4-4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BO or BODIPY), isothiocyanate, cyanines, or other fluorescent dyes. The interaction between a fluorescently labeled probe/ligand and a component of a biological sample can be detected by a spectrofluorimeter or preferably, by analyzing the fluorescence polarization of the mixture.

The binding interaction between a probe and a component of a biological sample may also be detected by ELISA (enzyme linked immunosorbent assay). The probe can be labeled or conjugated to such molecules as biotin, streptavidin or digoxigenin. Probes labeled with these molecules can be detected using enzyme conjugated antibodies specific for the label. Alternatively, the probe can be labeled with an antibody, which may or may not be conjugated to an enzyme. An antibody not conjugated to an enzyme can be detected by a secondary antibody that is conjugated to an enzyme. The enzyme conjugated antibody will react with an appropriate substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label an antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholinesterase.

The methods of the present invention may use ligand libraries synthesized according to any techniques known to those skilled in the art. Preferably, they are made using conventional solution phase reactions or solid phase synthetic techniques. Organic molecules of interests, such as biologically active compounds containing primary or secondary amine group, or hydroxyl groups, or thiol groups, or aldehydes or ketones, or carboxylic acids, can be labeled directly with suitable fluorescent molecules (dyes) in solution to give the corresponding fluorescent-labeled ligands. These methods and dyes are described in Haugland, R.P. Handbook of Fluorescent Probes and Research Chemicals, 6th Ed., 1996. In a preferred embodiment of the invention,

the solution phase syntheses are carried out in a suitable polar organic solvent or solvent mixture such as DMF, DMSO, THF using a slightly excess of dyes to ensure complete labeling. The resulting fluorescent-labeled ligands are purified by standard techniques in organic synthesis such as

5 liquid-liquid extractions using acid or base, crystallizations and chromatography (thin-layer or column). Alternative purification methods, such as liquid-solid phase extractions using polymer-bound scavengers to removal the unreacted dyes followed by simple filtrations can also be

10 used as described in the following examples (See, Obrecht, D. and Villalgordo, J.M., Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries, Pergamon, 1998, Chapter 3.)

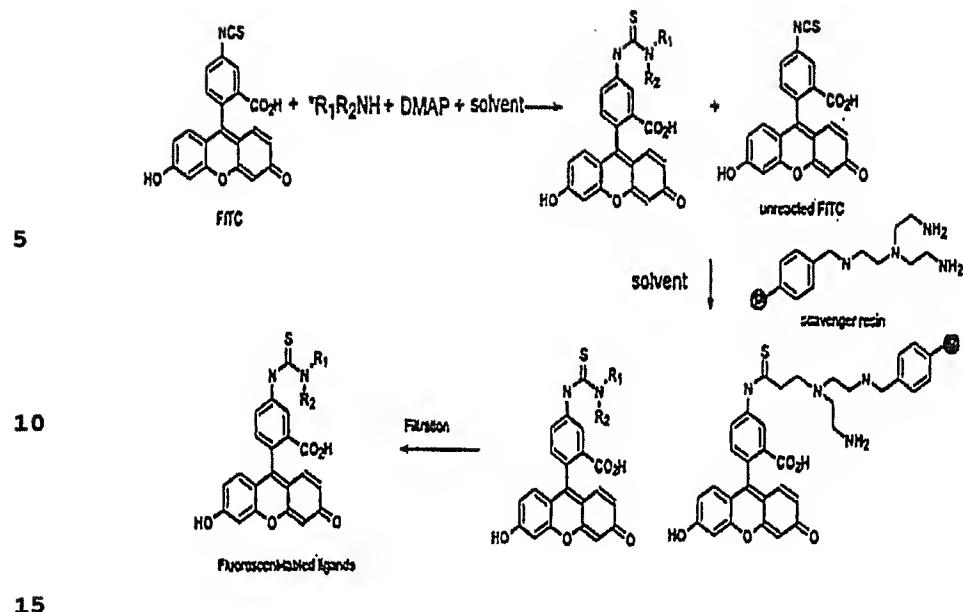
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Scheme I. Solution phase reactions (products were purified by scavenger resin)

20 In one embodiment of the present invention, the libraries of this invention are made using conventional solid phase techniques. See, e.g., Bodanszky, *Principles of Peptide Synthesis* (Springer-Verlag: 1984); Bodanszky, et al., 25 *The Practice of Peptide Synthesis* (Springer-Verlag: 1984); Barany and Merrifield *The Peptides: Analysis, Synthesis and Biology* Vol. 2, Chapter 1 (Academic Press: 1980); Atherton, et al., *Bioorg. Chem.* Vol. 8 (1979). This is because solid 30 phase synthesis has several advantages over more traditional synthetic methodologies. For example, large excesses of reagents or starting materials can be used to drive individual reactions to completion, and purification and isolation can be performed by simple filtration and washing 35 since the products are attached to solid supports. Furthermore, the relative site isolation of resin-bound species inhibits many types of intermolecular side reactions.

A solid phase synthetic method that has been found to be particularly suitable for the synthesis of the libraries of this invention is described below. Capable of quickly and efficiently forming diverse libraries of fluorescent-labeled ligands, this method comprises two general steps. In the first, a fluorescent dye is covalently attached to a solid

support. In the second step, which may be repeated as many times as necessary, the immobilized dye is reacted with a compound or mixture of compounds to form the desired mixture of ligands. The present invention encompasses assays using libraries adhered to the solid supports upon which they were made, or adhered to different solid supports. It is preferred, however, that the mixture of ligands be cleaved from the support in a third step. This optional third step is included in the preferred embodiment of the synthetic method of this invention shown in Scheme II:

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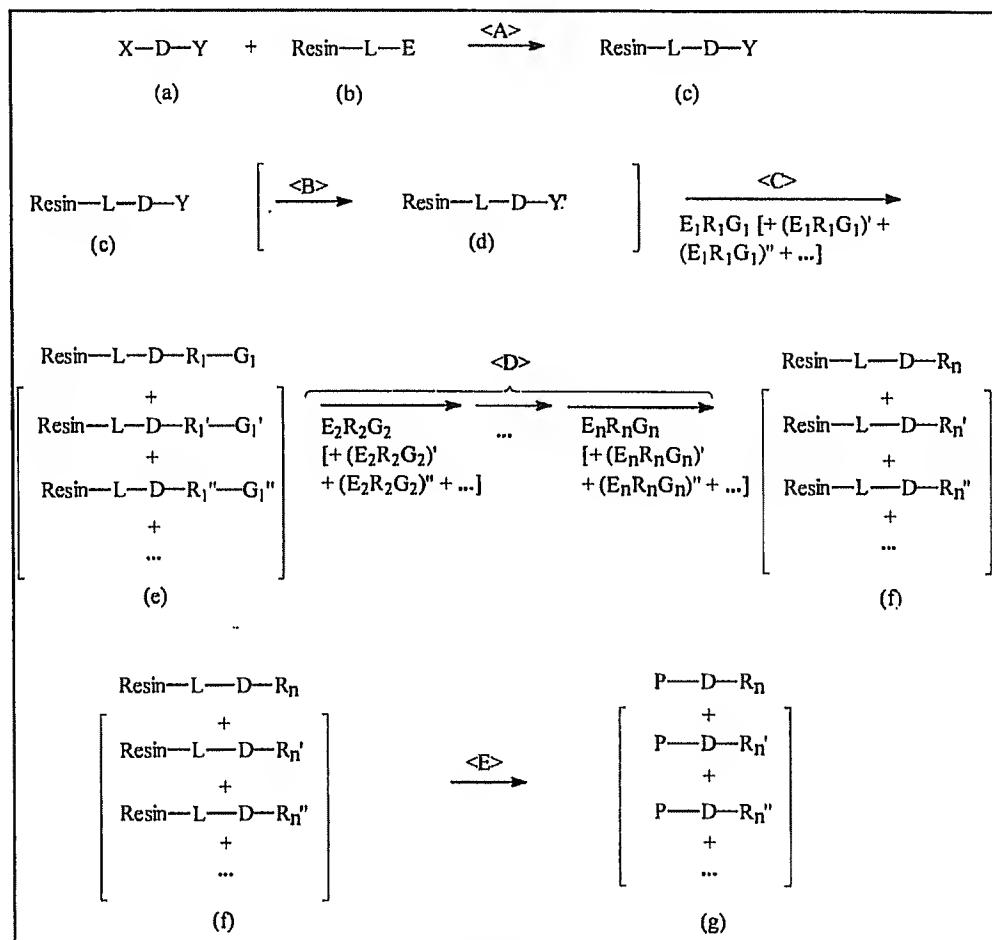
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Scheme II

wherein $\langle A \rangle$, $\langle B \rangle$, $\langle C \rangle$, $\langle D \rangle$ and $\langle E \rangle$ represent reaction conditions suitable for the formation of the desired products or intermediates represented by Formulas (b) - (g), and brackets (i.e., []) represent optional parallel or sequential reactions, reactants, and/or products.

According to Scheme II, a dye molecule of Formula (a) is selected:

X-D-Y
Formula (a)

5

wherein D is a fluorescent moiety, and X and Y are functional groups independently selected from the group consisting of halogens, alcohols, nitros, thiols, ethers, esters, carboxylic acids, α -halo carboxylic acid derivatives, amines, 10 amides, and protected and unprotected derivatives thereof. Examples of dye molecules of Formula (a) include, but are not limited to: fluorescein derivatives such as dichlorotriazylaminofluorescein (DTAF), dichlorosulfofluorescein (DCSF), and nitrofluorescein; 15 tryptophan derivatives; coumarin derivatives; napthyl derivatives; bipyridine (bpy) derivatives; tripyridine derivatives; cyanines; rhodamines and organometallic complexes such as Ru(bpy)₃, and derivatives thereof. The selection of dye molecules depends on a number of factors 20 including, for example, size, solubility, immunity to degradation under solid phase reaction conditions, absorption and emission wavelengths, quantum yield, and quantum yield and emission wavelength sensitivity to the surrounding chemical environment. Many of these factors, and the 25 synthesis of these and other suitable compounds, are readily determined from the literature. See, e.g., Haugland, R.P., *Handbook of Fluorescent Probes and Research Chemicals* (6th ed.; 1996).

30 Also according to Scheme II, a reactive substrate of Formula (b) is selected:

Resin-L-E
Formula (b)

35

wherein Resin represents any solid support suitable for solid phase synthesis; L is a linker attached to the solid support; and E is a leaving group bound to L. Suitable solid supports include, for example, polystyrene-divinylbenzene (PS-DVB) copolymer and polyethylene glycol-PEG-PS-DVB copolymer. Wang (polymer-bound 4-benzyloxybenzyl alcohol) and Rink resins, with and without suitable linkers attached, are available

from Aldrich Chemical Co., Milwaukee, WI; Novabiochem, San Diego, CA; and Advanced Chemtech, Louisville, KY.

A linker L-E is selected so that its bond to the solid support is readily cleaved under the reaction conditions represented by <E> in Scheme II. Suitable linkers are known to those skilled in the art and include, for example, halogens, thiols, alcohols, ethers, esters, aldehydes, ketones, carboxylic acids, nitros, amines, amides, silanes, and protected and unprotected derivatives thereof. The attachment of such linkers to solid supports may be accomplished by methods well known to those skilled in the art. See, e.g., Bunin, B.A., *The Combinatorial Index*, Academic Press, 1998.

In addition to the criteria described above, the linker L-E is selected so that it will form a covalent bond with the fluorescent moiety D of the dye molecule of Formula (a) under reaction conditions <A> to yield an immobilized dye of Formula (c):

20 Resin-L-D-Y
Formula (c)

Suitable reaction conditions <A>, which depend upon Resin, L, E and X, are well known to, or easily determined by, those skilled in the art. Generally they include the use of a 25 solvent that causes the resin to swell and react with X. Suitable solvents include, for example, dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), tetrahydrofuran (THF), CH_2Cl_2 , and mixtures thereof. The reaction conditions <A> also may include a base such as diisopropylethylamine 30 (DIPEA), triethylamine, dimethylaminopyridine (DMAP), or N-methylmorpholine (NMM), to neutralize the acid generated during the reaction.

The immobilized dye of Formula (c) serves as a foundation upon which the ligands of the library (represented 35 by Formula (g) in Scheme II) are formed. If the reactive moiety Y is protected, however, it must be deprotected prior to additional reactions. This optional deprotection to form the deprotected moiety Y' is performed under reaction conditions represented by in Scheme II. These conditions, which vary depending upon the protecting group, are well known to those skilled in the art. See, Greene,

T.W. and Wuts, P.G.M., *Protective Groups in Organic Chemistry* (2nd ed.; 1991).

The immobilized dye is then reacted under reaction conditions <C> with a compound of formula $E_1R_1G_1$ to yield a compound of Formula (d):

5

Resin-L-D-R₁-G₁
Formula (d)

10 wherein E_1 and G_1 may be the same or different, E_1 is a leaving group or protecting group, G_1 is either the terminal end of R_1 or a leaving group or protecting group, and R_1 represents any chemical fragment which comprises at least one protected or unprotected reactive moiety that enables the addition of R_1 to the fluorescent moiety D under suitable 15 catalytic and/or deprotecting conditions. Suitable reactive moieties include, but are not limited to, halogens, thiols, alcohols, ethers, esters, aldehydes, ketones, carboxylic acids, nitros, amines, amides, silanes, and protected and unprotected derivatives thereof. Suitable reaction 20 conditions <C> include those which have been developed for solid phase combinatorial chemistry. See, e.g., Brown, R., *Contemporary Organic Synthesis*, 216 (1997); Felder, E.R., and Poppinger, D., *Adv. Drug Res.*, 30:111 (1997); Balkenhol, F., 25 et al., *Angew. Chem. Int. Ed. Engl.* 35:2288 (1996); Hermkens, P.H.H., et al., *Tetrahedron* 52:4527 (1996); Hermkens, P.H.H., et al., *Tetrahedron* 53:5643 (1997); Thompson, L.A., et al., *Chem. Rev.* 96:555 (1996); and *Chem. Rev.* 97(2) (1997). 30 Exemplary addition reactions include that of a primary amine with an aldehyde to form an imine, which in turn can react with a variety of different moieties including, for example, β -lactams, pyrrolidines, thiazolidinones, and amides. Acid groups are equally flexible, and be used, for example, with 35 aldehydes, amines and isonitriles under Ugi multicomponent condensation conditions to form either small amides or heterocyclic compounds.

As indicated by Scheme II, the immobilized dye molecules of Formula (c) may also be reacted with a mixture of compounds, each of which is different but is of the general formula $E_1R_1G_1$; i.e., $E_1R_1G_1 + (E_1R_1G_1)' + (E_1R_1G_1)'' + \dots + (E_1R_1G_1)^i$, wherein i is the number of compounds in the mixture

and is an integer preferably less than about 50. In such a case, a mixture of compounds of Formula (d) is produced, each possessing a different R_1G_1 fragment; i.e., Resin-L-D- R_1G_1 + Resin-L-D-(R_1G_1)' + Resin-L-D-(R_1G_1)'' + ... + Resin-L-D-(R_1G_1)ⁱ.

5 It is preferred, however, that the compounds of Formula (c) only be reacted with one compound of formula $E_1R_1G_1$.

Because many pharmacologically active compounds contain reactive moieties such as amines and carboxylic acids, the present invention contemplates that such compounds are encompassed by the formula $E_1R_1G_1$, in which case further 10 reaction may or may not be desired. If, however, the R_1 fragment of the compound(s) of Formula (d) is a reactive moiety, $n-1$ subsequent addition reactions may be performed under reaction conditions that are collectively referred to in Scheme II by <D>, wherein n represents the number of 15 moieties bound to the fluorescent moiety D, and is an integer of preferably less than about 100.

As above, each of these subsequent addition reactions 20 may employ both single compounds or mixtures of compounds of formulas $E_kR_kG_k$, wherein k is an integer between 2 and $n-1$, R_k is the k^{th} moiety bound to the immobilized fluorescent moiety D (via the $k-1$ moieties already bound to D), E_k and G_k are the same or different, E_k is a leaving group or protecting group, 25 G_k is the terminal end of R_k or a leaving group or protecting group, and R_k represents any chemical fragment which comprises at least one reactive moiety that enables the addition of R_k to the immobilized compound(s). Suitable reaction conditions <C> include the use of catalysts, deprotectants, and the like which facilitate the addition of R_k to the immobilized fluorescent compounds.

30 Completion of the reactions described above forms either an immobilized compound of Formula (f):

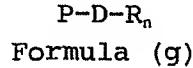
Resin-L-D- R_n
35 Formula (f)

or a mixture of immobilized compounds of Formula (f); i.e., Resin-L-D-($R_1R_2R_3\cdots R_n$) + Resin-L-D-($R_1R_2R_3\cdots R_n$)' + Resin-L-D-($R_1R_2R_3\cdots R_n$)'' + ... + Resin-L-D-($R_1R_2R_3\cdots R_n$)^m, wherein m has a maximum value of about $i*n$ when i is equal to the number of compounds in the $E_kR_kG_k$ mixture having the largest number of compounds. For the sake of simplicity, G_n is omitted from

Formula (f) because the terminal end of the ligand (e.g., R_n) undergoes no further addition reactions.

In the final step of Scheme II, the dye-ligand compounds are cleaved from the solid support under reaction conditions <E> to yield a library of compounds of Formula (g):

5



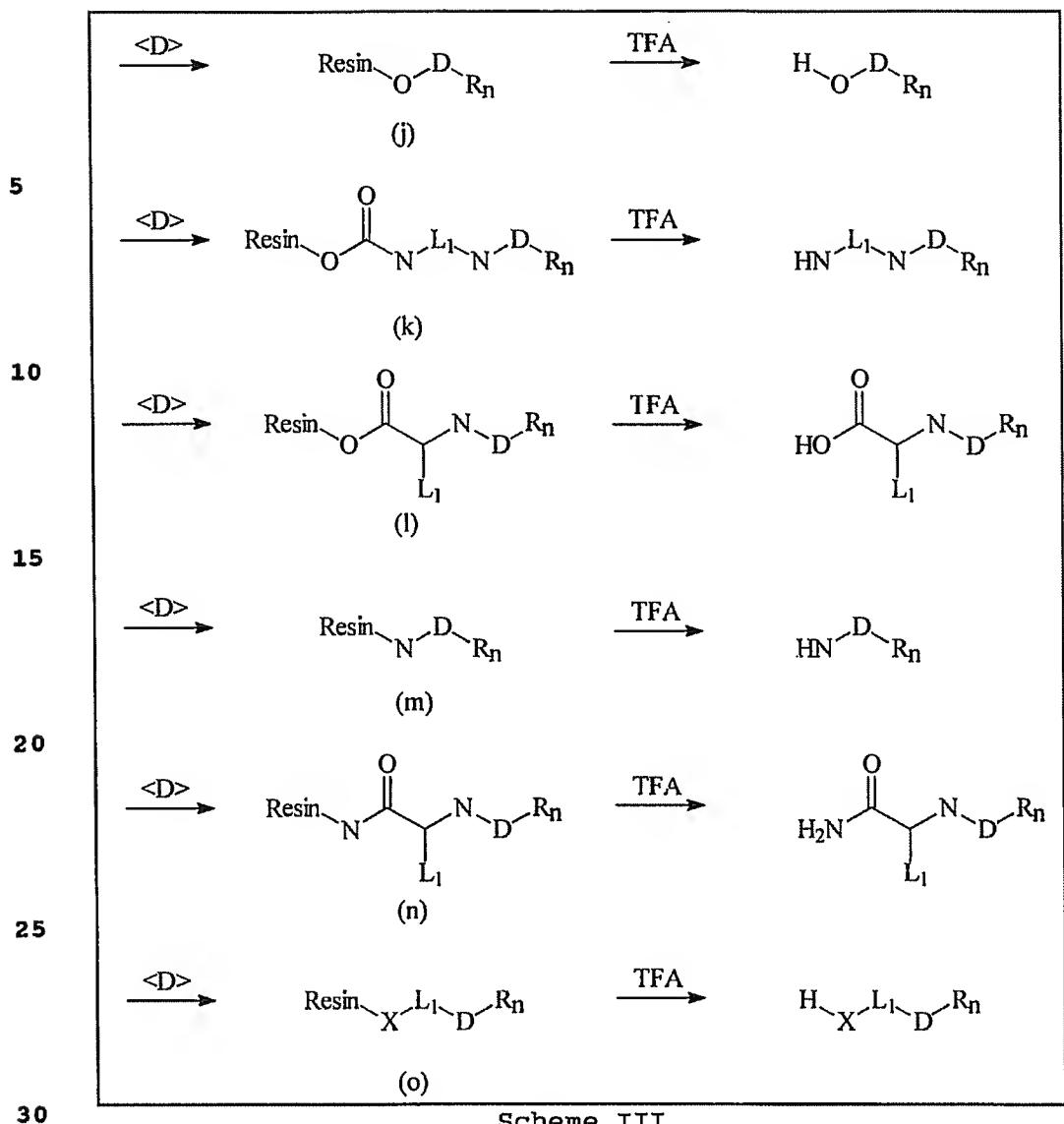
wherein it is to be understood that Formula (g) encompasses all possible compounds and mixtures of compounds produced by the reactions indicated in Scheme II. Suitable cleavage conditions <E> are known to those skilled in the art, and depend upon the bond between Resin and L. Cleavage may be accomplished under acidic or basic conditions, or may be photoinduced. Many suitable cleavage methods have been reported in the literature. For example, some cleavage reactions accomplished by treating the modified resin of Formula (f) with trifluoroacetic acid (TFA) in methylene chloride are shown in Scheme III:

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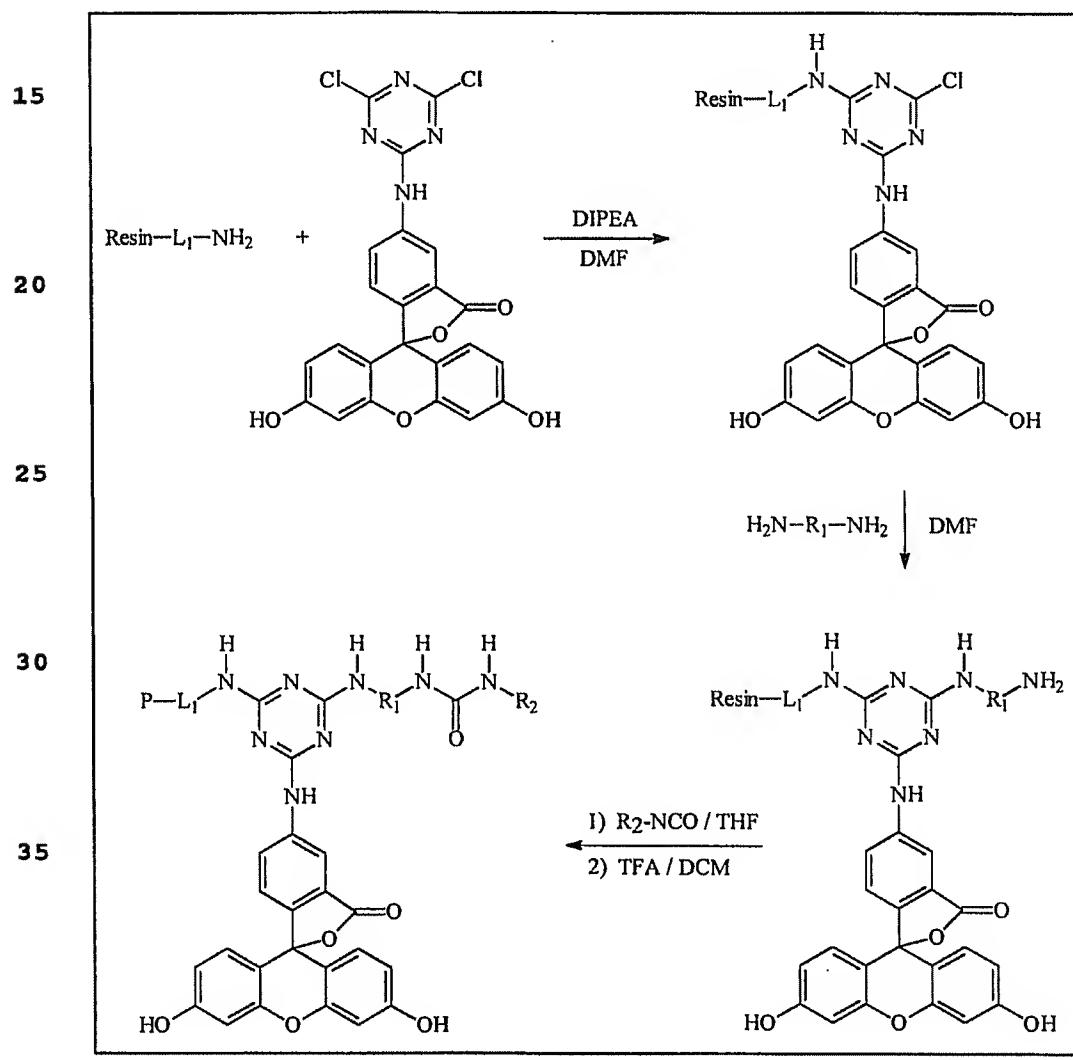
Scheme III

wherein (j) is a Wang resin derivative; (k) is a Wang carbamate resin derivative; (l) is a Wang amino acid resin derivative; (m) is a Rink resin derivative; (n) is a Rink amino acid resin derivative; and (o) is a trityl or chlorotriptyl amine (i.e., X= NH) or alcohol (i.e., X=O) resin derivative; and L₁ represents any side chain or spacer stable under solid phase reaction conditions. Examples of suitable side chains include, but are not limited to, substituted and unsubstituted alkyl, aryl and aralkyl groups.

After cleavage, the solvent is preferably removed to isolate the fluorescent library. The library may then be dissolved in a solvent such as dimethylsulfoxide (DMSO) suitable for use in the assays of this invention.

Specific embodiments of the method of Scheme II are shown in Schemes IV - VIII. For clarity, these schemes do not show the reaction and formation of mixtures. It is to be understood, however, that each of the individual reactions shown represents the possibility of numerous parallel reactions.

A particular simplified embodiment of the general approach of Scheme II is shown in Scheme IV:



wherein L_1 is any moiety that does not sterically hinder or otherwise inhibit the coupling reaction under the reactions conditions shown; P represents the end of L_1 after it has been cleaved from the solid support; and R_1 and R_2 are the same or different and may be any moieties desired to provide a

5 library with preferred structural and reactive characteristics. Examples of suitable moieties include, but are not limited to, substituted and unsubstituted alkyl, aryl and aralkyl.

According to Scheme IV, DTAF is immobilized upon a solid

10 support using diamino carbamate Wang resin or amino acid Rink resin or diamino/amino alcohol trityl/chlorotriyl resin to give monochlorotriazylaminofluorescein resin. This reaction is preferably conducted at ambient temperature. DTAF is dissolved in a suitable solvent such as DMF, NMP, THF,

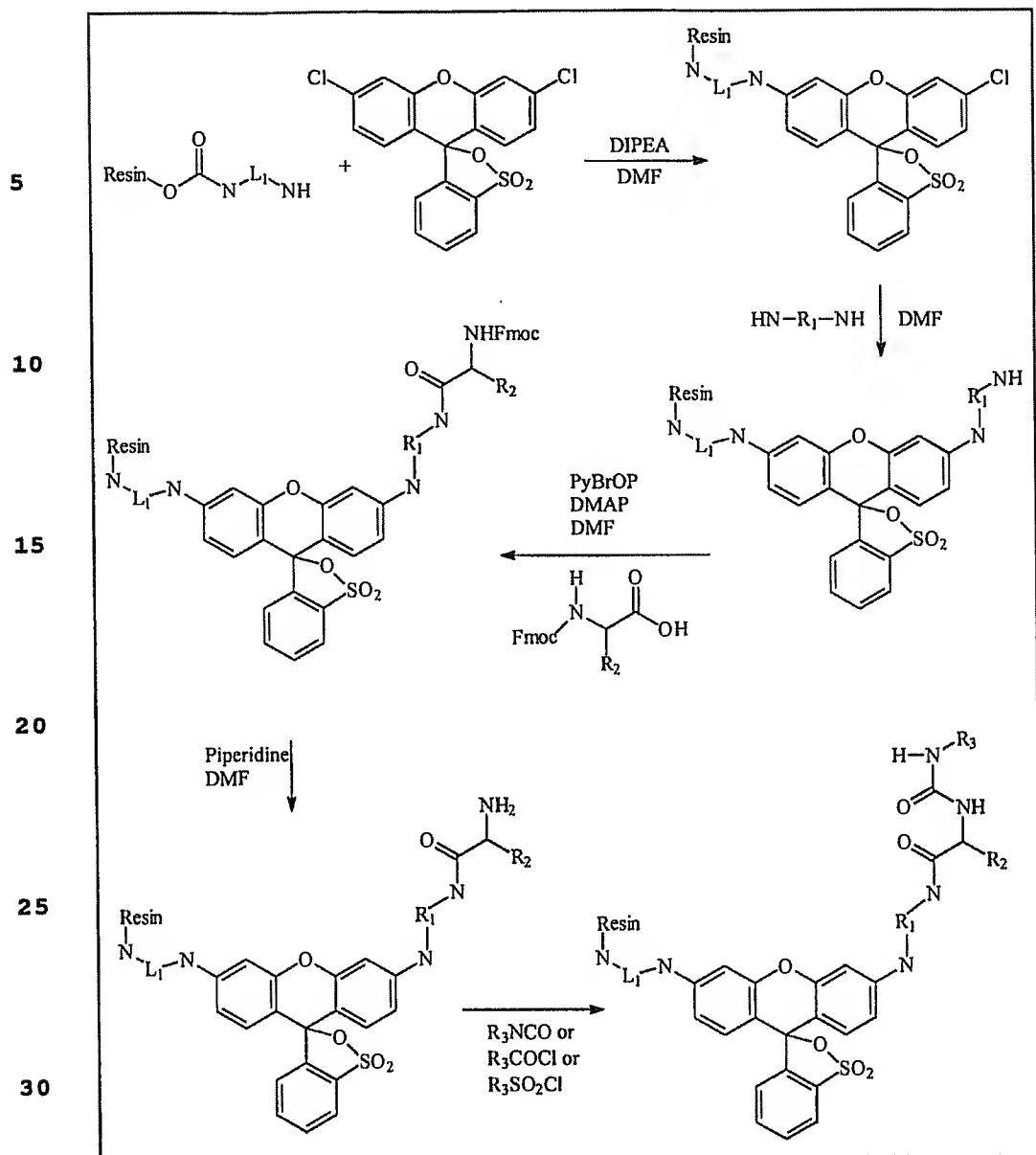
15 methylene chloride, or mixtures thereof with between about 0.5 to about 3 equivalents of a base such as DIPEA, triethylamine, DMAP, or NMM. Substitution of the remaining chloride on the triazine ring with an excess of symmetrical diamine (preferably between about 2 to about 6 equivalents)

20 in, for example, DMF or NMP at ambient temperature provides a new reactive group for further synthesis. This process is repeated as many times, and with as many different reactants, as desired, after which the resulting fluorescent compound or mixture of compounds is cleaved from the reactive support.

25 Another embodiment of the general approach of Scheme II is shown in Scheme V:

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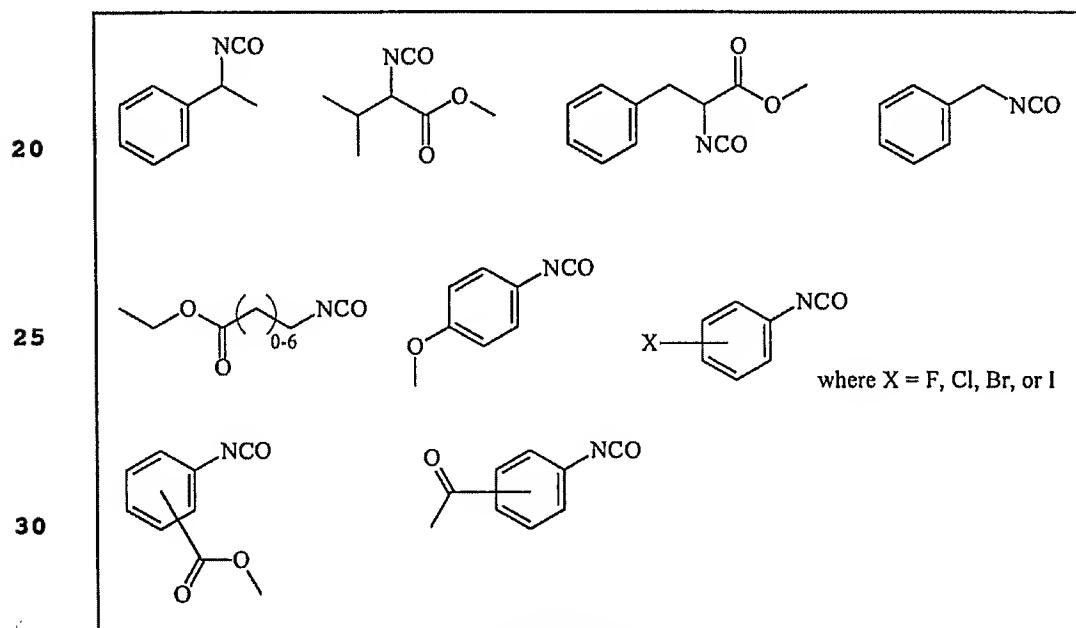
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35 wherein DCSF is attached to a solid support via substitution of a chlorine atom by a secondary alkyl amine, and preferably a cyclic secondary amine, bound to a resin. L_1 thus forms part of a cyclic diamine. Suitable cyclic diamines include, for example, piperazine, homopiperazine, 4,4'-trimethylenedipiperidine, and derivatives and isomers thereof. As shown, R_1 also forms part of a cyclic diamine, although HNR_1NH may be replaced by any compound having

suitable reactive groups. R_2 and R_3 represent any moieties suitable for incorporation within the fluorescent ligands of the libraries of this invention and include, for example, the side chains of natural amino acids; substituted and unsubstituted alkyl, aryl and aralkyl; and the like.

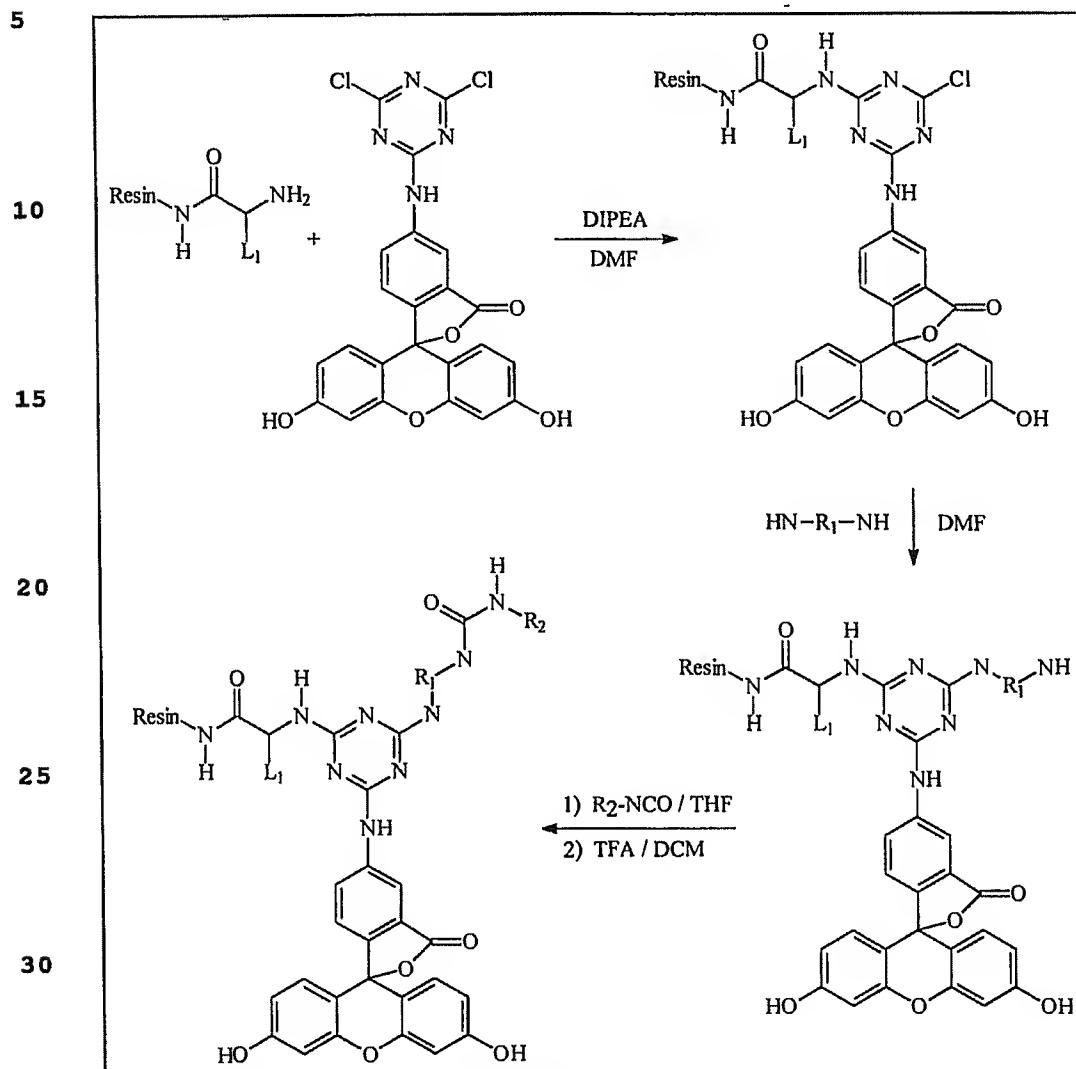
5 As shown in Scheme IV, an N-Fmoc protected amino acid is attached to the fluorescent resin by the reaction of the free amino group of the fluorescent compounds with the Fmoc amino acid under standard amide formation conditions (i.e., PyBrOP/DMAP/DMF). After removal of the Fmoc group with 10 piperidine in DMF, the new amino group provided by the amino acid can be derivatized with, for example, acid chlorides, chloroformates or isocyanates to give a variety of fluorescent labeled compounds. Non-limiting examples of 15 suitable isocyanide compounds are provided in Scheme VI:



35 As should be readily apparent to those skilled in the art, numerous other moieties (i.e., R_4 , R_5 , ..., R_n) comprising reactive moieties such as amino acids, acid chlorides, chloroformates, and isocyanates may be used to form the ligands bound to DCSF. Similarly, the chemical fragments to which the R_2 and R_3 groups of Scheme IV are attached may be replaced by any others which will allow the growth of the

chain bound to the dye molecule provided that the reaction conditions are altered in a suitable manner.

A final embodiment of the general approach of Scheme II is shown in Scheme VII:



35 wherein DTAF is bound to a Rink amino acid resin, and then subsequently derivatized by the methods described above. L₁, R₁ and R₂ are defined as above.

In addition to the above methods, fluorescent-labeled ligand libraries are also made by general solid-phase synthesis techniques (Obrecht, D. and Villalgordo, J.M., Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries, Pergamon, 1998;

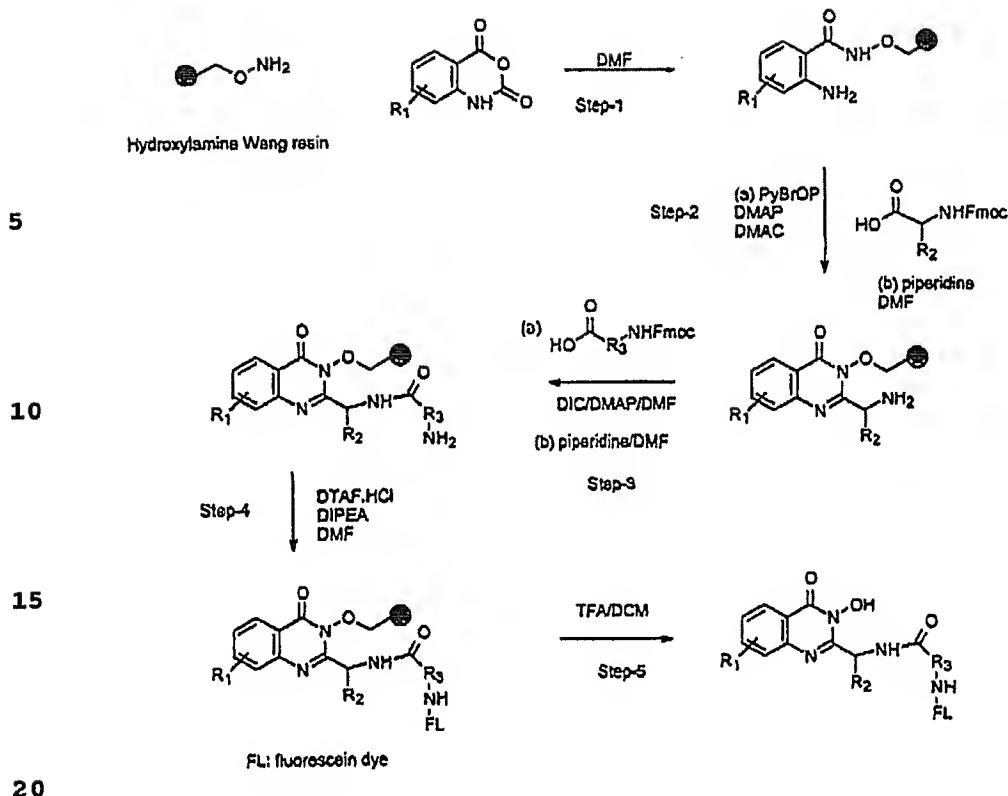
and Bunin, B.A., *The Combinatorial Index*, Academic Press, 1998). In a preferred embodiment of the present invention, the desired compounds are synthesized on the solid supports according the methods described in the literature. Before cleavage from the solid supports, the compounds on the solid
5 supports are treated with suitable dyes to give the fluorescent-labeled ligands on resins. The ligands are then cleaved from the resins to give the fluorescent-labeled ligands.

In one approach, the ligands are synthesized in a linear
10 fashion by reaction of a solid supported reactive block with different reactive blocks step by step. The dye is then added in the last step before cleavage to give the labeled ligands as shown in Scheme VIII. In this scheme, fluorescent-labeled N-hydroxyquinzolinones are prepared.
15 Quinzalinones are one of the most common bioactive nitrogen containing heterocycles (See, Sinha, S. and Srivastava, M. in *Progress in Drug Research*, 1994, vol. 43, 143-238). They display a broad spectrum of biological and pharmacological activities in human and animals. They have been used as
20 anticonvulsant, antibacterial and antidiabetic agents. Therefore, fluorescent-labeled quinzolines would be useful for diagnostic applications and for drug discovery.

25

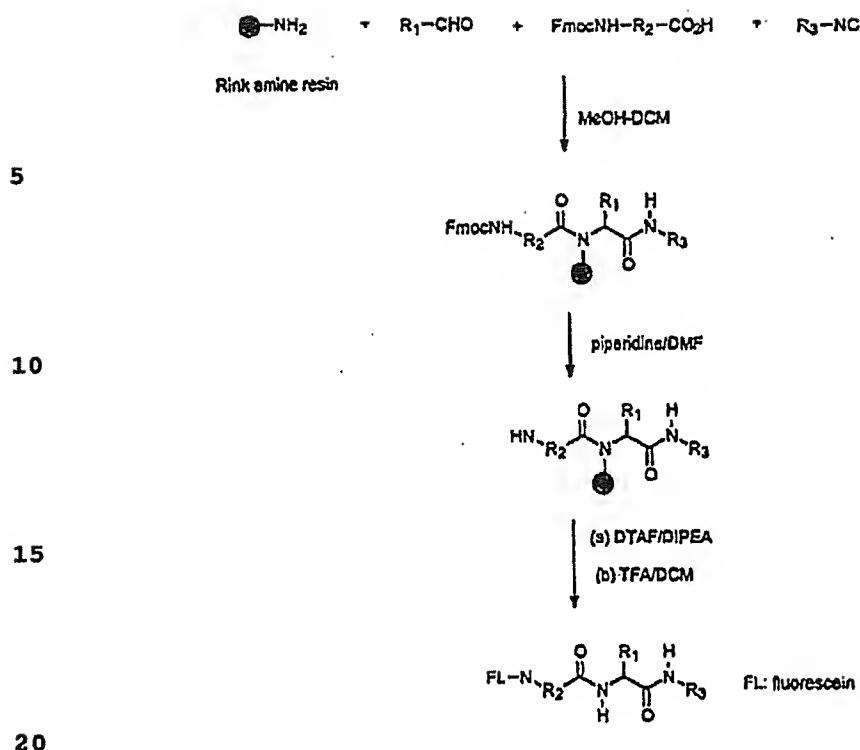
30

35



Scheme VIII

25 In another approach, the ligands are prepared in a convergent manner using multicomponent condensation reactions such as the Ugi condensation (Tempest, P.A., et al. *Angew. Chem. Int. Ed. Engl.* 1996, vol. 35, 640-642). Using this 30 approach, the amine component is immobilized on the solid support as Rink amine resin and the aldehydes, Fmoc-protected amino acids, and isocyanides are added in excess to the resin swelled in mixture of MeOH/DCM (1:2 v/v) (Scheme IX). By 35 combinatorial uses of different aldehydes, acids and isocyanides, a large number of ligands may be synthesized.



Scheme IX

5.2 BIOLOGICAL SAMPLES

25 The present invention provides methods for "fingerprinting" complex biological mixtures or samples which may be obtained from a wide variety of sources. The methods of the present invention may be utilized to identify specific phenotypical differences which exist between normal or

30 abnormal, healthy or diseased, nontransformed or transformed, noninfected or infected cells and/or tissues. The methods of the present invention may also be applied to identify or distinguish between species of microorganisms, viruses, bacteria, fungi or parasites.

35 The methods of the present invention detect all types of ligand/receptor interactions, whereby the receptor could be proteins, carbohydrates, nucleic acids, or any molecules with a shape that is capable of interacting with a probe or ligand. As used herein, "biological receptor", "receptor", "biological target", "target" and "component of a biological sample" are intended to include any biological molecule, binding surface, binding site or the like, that is for

example, differentially expressed, or is differentially modified in the test sample, e.g., the diseased or otherwise abnormal cells and/or tissues. Any one of these receptors may serve as a target for diagnosis and/or development of potential therapeutics.

5 Accordingly, one aspect of the present invention is a method for characterizing a pathology, said method comprising identifying a pattern of binding interactions between receptors or targets present in biological samples associated with the pathology, and a library of ligands or probes,
10 wherein the pattern of binding interactions provides a unique fingerprint for the pathology.

In accordance with the present invention, a "receptor" or "target" is a biological molecule which demonstrates a binding affinity or interaction with a library of ligands or
15 probes in the test sample which may or may not differ between the test sample and the control sample. Examples of such receptors or targets include, but are not limited to, proteins, including enzymes, antigens, antibodies, lipids, nucleic acids including DNA and RNA, carbohydrates including
20 lectins, cell surface proteins or receptors, etc.

The biological samples utilized in the method can be any sample that is a source of biological molecules, including but not limited to, biological materials such as body fluids, such as blood plasma, blood sera, urine, cerebrospinal fluid,
25 amniotic fluid, saliva, mucous, tissue samples, cell extracts, products from *in vitro* transcription and translation systems (obtained, for example by the method of U.S. Patent 5,654,150 issued to King et al.), and the like.

30 Moreover, extracts derived from or fluids containing pathogenic organisms such as bacteria, yeast, fungi, viruses, protozoa, and the like may also be used. In these instances, ligands exhibiting high affinity and specificity for a protein or other receptor in the pathogen may reveal new
35 targets and can be tested for inhibitory effect against the pathogen.

The biological samples which may be screened in accordance with the methods of the present invention may be obtained from a wide variety of sources. By way of example, but not by limitation, biological samples or mixtures may be obtained from patients and include bodily fluids, blood, serum, mucous, including oral, rectal or intestinal mucosa,

urine, feces, etc. In addition biological samples may include tissue samples, biopsy tissue, cell samples, including bone marrow cells, lymphocytes, immune cells, mucousal cells obtained from oral, rectal or intestinal mucousal linings, etc. In yet another embodiment, the 5 biological samples or mixtures may encompass cell lysates or portions thereof, carbohydrates including lectins; proteins including glycoproteins, cell surface receptors, peptides; nucleic acids including DNA or RNA etc. In yet another embodiment the biological sample may be or may be derived 10 from a virus, bacteria, microorganism or parasite or fluids containing such biological samples, e.g., testing water supplies for microorganism content.

The biological samples of the present invention may be obtained from individuals inflicted with a disease, disorder, 15 or pathology infected with a virus, bacteria or other microorganism. In yet another embodiment, the biological samples may be generated by exposing a tissue, cells in culture, cell extracts etc. to a toxin or pathogenic agent, or by genetically engineering the genome of a cell in culture 20 to encode a mutation or protein or peptide known to be associated with any given pathology or disorder.

Collections of biological materials as sources for clinical samples may be obtained from hospitals or national research facilities.

25

5.3 ASSAYS FOR THE DETECTION OF LIGAND/RECEPTOR INTERACTIONS

Recognizing that clinical materials are often available in only a limited supply for any given pathology, the third 30 element of the invention, the sensitive assay systems, must be capable of detecting binding interactions occurring in only microliters of sample and in addition, must be capable of detecting binding interactions of less than optimal affinity. The assay systems of the present invention 35 eliminate or greatly reduce nonspecific binding of ligands to receptors present in the sample.

Accordingly, an aspect of the assays of the present invention is to provide a means for detecting the interaction between the probe or ligand and the receptor, target or active site. In one such embodiment, homogeneous assays are utilized for the detection of receptors or targets present in a biological sample. The assays comprise contacting a

biological sample with a library of diverse probes in a microtiter dish or other well-type device and detecting any binding of individual members of the library of probes to components in the system. The binding pattern of members of the library of probes with the sample provides a molecular 5 fingerprint of the sample. In a preferred embodiment, the binding of a member of the library to a component of the sample is detected by fluorescence polarization.

In another embodiment, the assay for detecting receptors or targets present in a biological sample encompasses 10 attaching the probes and ligands of the library to a structure which contains a site cleavable by a specific catalyst, the cleavage of the structure or scaffold provides the signal or the means for detecting the interaction between the probe and the receptor. More particularly, the members 15 of the library of ligands or probes are attached to scaffolds having a site cleavable by a specific catalyst and a marker on each side of the site, to provide ligand/scaffold constructs; the ligand/scaffold constructs are contacted with a biological sample, wherein a ligand having affinity for a 20 receptor present in the sample binds with the receptor and blocks the cleavable site of the scaffold. Once the ligand/scaffold construct is contacted with the specific catalyst, the scaffolds that do not include a bound receptor are cleaved and the intact scaffolds are identified.

25 A preferred embodiment of the assay further comprises attaching a marker, such as biotin, to the same side of the scaffold as the cleavable site and attaching a second marker, such as digoxigenin, to the opposite side of the scaffold. The scaffold is immobilized on a surface, and the mixture is 30 washed after the addition of the catalyst to remove the portions of cleaved scaffolds, portions including the second marker, so that the intact scaffolds are identified by the presence of the second marker.

The scaffolds used in the assay may be comprised of DNA, 35 RNA, peptides, peptoids, oligosaccharides or any hydrophobic or hydrophilic, synthetic or natural polymer including block copolymers. In a preferred embodiment, the scaffolds are double-stranded DNA, constructs comprised of a first oligonucleotide, a second complementary oligonucleotide having a ligand attached thereto; and a single restriction site. In this embodiment, the specific catalyst is the restriction enzyme specific for the restriction site. The

first marker can be, for example, biotin, whereby the ligand structures can be immobilized on a streptavidin or avidin coated surface. Digoxigenin is a preferred second marker, whereby anti-digoxigenin-peroxidase is used to indicate the presence of intact scaffolds.

5 Another assay for detecting the presence of receptors in a biological sample consists of attaching members of a library of ligands to a scaffold, contacting the scaffold with a scaffold-specific binding agent and a biological sample, and detecting the interaction between a ligand and a receptor present in a biological sample. The scaffold used in this assay may be comprised of DNA, RNA, peptides, peptoids, oligosaccharides or any hydrophobic or hydrophilic, synthetic or natural polymer including block copolymers. In a preferred embodiment, the scaffold-specific binding agent 10 does not bind to the region of the scaffold where the interaction between a ligand and a receptor occurs. The scaffold-specific binding agent may be a drug, peptide, glycoprotein, protein, polysaccharide, saccharide, or inorganic molecule. In another embodiment, the scaffold 15 includes a marker that is blocked when the scaffold-specific agent binds to the scaffold such that access to the marker indicates the absence of the scaffold-specific binding agent and the presence of the ligand/receptor interaction. In a preferred embodiment, the scaffold is comprised of double- 20 stranded DNA and the marker is a biotinylated nucleotide, which is detected with streptavidin or avidin when a receptor/ligand interaction occurs.

In still another embodiment, the principles of the present invention may be used for the toxicological screening 30 of potential pharmaceuticals. At present, the prediction of toxic properties, or side effects, is difficult during the early stages of drug development that occur prior to clinical testing. By utilizing the principles of the present invention, the presence or absence of toxic properties may be 35 predicted at a much earlier stage in development than is currently possible.

In accordance with this aspect of the invention, cultured cells or tissues are treated with known toxic or non-toxic compounds, and then extracts are prepared from the cultures and probed with a library of labeled compounds. This probing with the library of labeled compounds provides a "fingerprint" patterns which reflects the responses of the

cells or tissues to the toxic or non-toxic compounds. Any changes in the fingerprint patterns correlated with toxic properties are noted, and perhaps further classified using statistical analysis or analysis by artificial intelligence routines (neural networks). Subsequently, cultured cells or 5 tissues for which a fingerprint pattern has been established as set forth above are treated with a test compound having unknown toxic properties, and extracts are prepared from the treated cultures and probed with the library of labeled compounds. The resulting fingerprint or profile of the 10 cell's or tissue's responses is then compared with the reference profiles generated for that cell or tissue using the known toxic/non-toxic compounds to predict the nature of the test compound.

In accordance with the present invention, sections 5.3.1 15 through 5.3.3 describe the assays that may be utilized to detect the differences in binding affinities of probes to biological samples such that a fingerprint or pattern of binding affinities is generated.

The results obtained with the present invention serve to 20 differentiate two sample populations (e.g., normal and diseased) and can be expected to fit into two categories. In the first category, one or two probes can serve to differentiate the populations and the probe response will be easily distinguishable between the two groups ("shining 25 pebbles"). In the second category, subtle difference in binding to two sample populations can be differentiated using a greater number of probes, which have a small difference in binding to the sample populations. The combination of these probes (a diagnostic cluster) will be necessary to provide 30 the statistical power for a reliable differentiation in unknown samples. A larger number of probes, is expected to result in a more useful analytical system since the overall binding pattern will not be severely affected by fluctuations due to individual variation. In a system where only one or 35 two probes are used, individual variation in the probes might make diagnosis more difficult.

Analysis of the binding of a large number of probes to a large number of samples requires the use of sophisticated computer algorithms. Two types of analysis can be performed on the data. The first type of analysis focuses on determining which probes are useful for distinguishing members of two sample populations. Algorithms for this type

of analysis are available from either the statistical analysis field (discriminate function analysis) or from the field of artificial intelligence (supervised, back-propagation neural networks). The second type of analysis that can be performed involves categorizing the probe binding data based upon the probe results and then asking if there are any similarities in the histories (e.g., medical) in the respective probe-based categories. Algorithms exist in statistical analysis (principal component analysis) and in artificial intelligence (unsupervised, Kohonen neural networks) for these types of analysis. Data analysis programs or packages are available commercially which include the above functions. For example, the commercial programs SPSS (SPSS, Inc., Chicago, Illinois) and SAS (SAS Institute, Inc., Cary, NC) provide discriminate functions analysis and principal component analysis. Several packages such as Neuroshell 2 (Word Systems Group, Inc., Frederick, MD) or the Stuttgart Neural Network Simulation (The University of Stuttgart, Stuttgart, Germany) provide back-propagation and Kohonen neural networks.

20

5.3.1 FLUORESCENCE POLARIZATION ASSAY

In the present invention, a fluorescence polarization assay can be utilized to identify binding interactions between a ligand and a receptor present in a biological sample.

A fluorescence polarization assay is designed to measure the binding of a fluorescent-labeled compound to an unlabeled biomolecule. A fluorescence polarization-based assay can utilize fluorescence labeled compounds up to a molecular weight around 10,000 to detect interactions with biomolecules. The type of fluorescent labeled compounds that can be used include, but are not limited to, small organic molecules, peptides, small proteins, nucleic acids, lipids, and polysaccharides. Fluorescent molecules when excited with plan polarized light, will emit light in a fixed plane only if they do not rotate during the period between excitation and emission. The extent of depolarization of the emitted light will depend upon the amount of rotation of the molecules, which is dependent upon the size of the molecule. Small molecules rotate more than larger molecules between the time they are excited and the time they emit fluorescent light. The optimum conditions of this assay exist when the

labeled compound is much smaller than the unlabeled molecule to which it binds. An unbound small fluorescent-labeled compound rotates rapidly and the emitted light is depolarized. The interaction between a biomolecule and a fluorescent-labeled compound increases the effective size of 5 the fluorescent-labeled compound and thus, decreases the rotation of the compound, which results in the emitted light remaining polarized. The intensity of emitted, polarized light can be measured by inserting a moveable polarizing filter in front of the detector. The intensities are 10 measured in planes 90° apart and are many times designated the horizontal and vertical intensities. In some instruments the excitation filter is moveable while the emission filter is fixed. In certain other machines the horizontal and vertical intensities are measured simultaneously via fiber 15 optics. Three companies, Pan Vera, BMG Lab Technologies, and LJI Biosystems, market research grade fluorescence polarization instruments and Abbott provides clinical laboratory instrumentation. The value of fluorescence polarization is determined by the following equation:

20

$$\text{polarization} = \frac{\text{intensity}_{\text{vertical}} - \text{intensity}_{\text{horizontal}}}{\text{intensity}_{\text{vertical}} + \text{intensity}_{\text{horizontal}}}.$$

Fluorescence polarization values are most often divided by 25 1000 and expressed as millipolarization units (mP). In one form of such an assay, a fluorescent molecule, such as fluorescein, is attached to an oligomer of sufficient length that the "propeller effect" comes into play. A short linker is then attached to the fluorescent molecule and the 30 end of the oligomer opposite the fluorescent molecule is attached to the walls of a microtiter dish or other well-type device suitable for use in fluorescence polarization. The free end of the short linker is then derivatized with ligands to be screened. The identity of the attached ligands in each 35 well is known. A source of receptors is then added to each well and incubated. Polarized light is then used to excite the fluorescent molecule, and the polarity of emitted polarized light is determined. Receptors that bind to the ligand will reduce the free rotation of the fluorescent molecule and increases in polarization will be detected. On the other hand if no receptor binds to the ligand, the fluorescent molecule remains freely rotating and the emitted

light will be less is not polarized. To obtain an approximation of the affinity of any bound receptor for the ligands, the extracts are removed from the wells, fresh buffer added and the polarization of light emitted from each well determined. Those receptors with low affinity will 5 dissociate from the receptor and the polarization of emitted light will decrease. Repeating this process will identify ligand/receptor binding interactions of the highest affinity.

5.3.2 SCINTILLATION PROXIMITY ASSAY (SPA)

10 Typically, in SPA assays the receptor is bound to a scintillant loaded bead and screening is done directly or competitively against ligands in solution. It is possible, however, to reverse this arrangement and have the ligand attached to the bead and have the receptor in solution. This 15 variation is especially compatible with combinatorial chemistry because in many synthetic schemes, the library synthesis is done on beads, which may subsequently be impregnated with scintillant as needed by an SPA assay system. In addition, more than one ligand can be synthesized 20 on a bead. In such a system, beads loaded with scintillant and coated with a ligand are immersed in a fluid phase containing radiolabelled reactant. If the labeled reactant has affinity for a tagged ligand and the two become bound, the resulting proximity of the radiolabelled reactant and the 25 scintillant in the beads leads to activation of the scintillant and the emission of light. If the labeled reactant has little or no affinity for a ligand, the radiolabel will not accumulate sufficiently close to the scintillant to allow for energy transfer following 30 radioactive decay. Because SPA does not require a washing step, it allows for the detection of relatively low affinity ligand/receptor binding interactions.

5.3.3 DNA RESTRICTION SITE OBSTRUCTION ASSAY

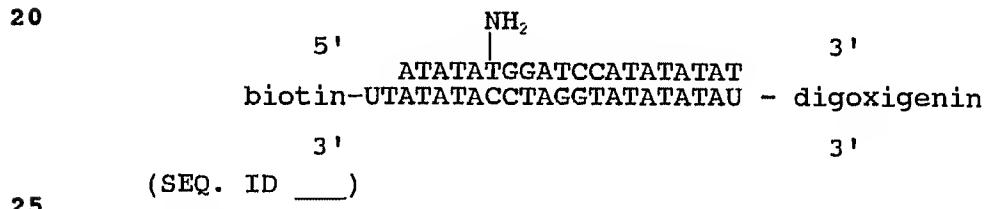
35 The principles of this system are based upon the presence or absence of restriction enzyme activity upon a DNA construct that has been synthesized to include a single restriction site and a ligand-reporter system. When the construct is contacted with a biological mixture, ligand/receptor interactions will block access of the restriction site by the restriction enzyme and prevent hydrolysis of the construct at the site. Constructs that

remain intact can be isolated and the ligand/receptor interaction identified.

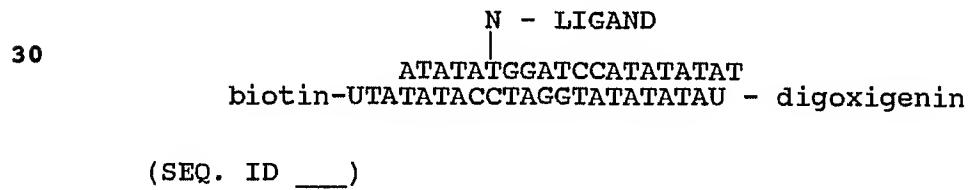
A description of a DNA restriction site assay system is provided herein. A single stranded DNA oligonucleotide containing biotin at the 5' end and digoxigenin at the 3' end 5 is annealed to a complementary oligonucleotide such that the annealed, double stranded oligonucleotide contains a single centrally located restriction endonuclease site. The complementary oligonucleotide is modified to contain a linker having a terminal amino group. The location of the amino 10 group, in this case between the 5th A and the 6th T from the 5' end, is such that it does not interfere with the activity of the restriction enzyme toward the double stranded oligonucleotide. The resulting construct, wherein the

15 sequence GGATCC (SEQ. ID) is a restriction site, is

shown below:



The amino group is derivatized with ligands of a diverse chemical library to form the construct shown below:



35 The derivatization of the amino group can be accomplished during synthesis of the single-stranded oligomer while it is still attached to the CPG bead, and alkaline cleavage then used to release the oligomer from the bead where it can then be annealed to the complementary biotin-digoxigenin oligomer. As an alternative method of attaching the ligands, derivatized bases, may be incorporated during the synthesis of the complementary oligonucleotide.

When incubated with the restriction enzyme specific for the restriction site, according to procedures well known in the art, the derivatized construct is hydrolyzed at the restriction site to provide two sections as shown below:

5 N - LIGAND

ATATATG
biotin-UTATATACCTAG + GATCCATATATAT
GTATATATAT - digoxigenin

10 (SEQ. ID ____ and ____)

Reaction of the hydrolyzed mixture with a streptavidin or avidin coated surface will result in the immobilization of the biotin labeled section and the digoxigenin labeled section will be eliminated by washing. Reaction of the immobilized mixture with anti-digoxigenin-peroxidase antibody will provide a negative result because the digoxigenin labeled section will have been eliminated from the mixture by hydrolysis by the restriction enzyme and subsequent washing.

When the intact ligand-derivatized construct functioning as a probe for detecting potential receptors within the 20 sample, is mixed with a clinical sample, the ligand will capture any receptor for which it has high affinity for as shown below:

25 N -LIGAND - RECEPTOR

ATATATGGATCCATATATAT
biotin - UTATATACCTAGGTATATAU - digoxigenin

(SEQ. ID ____)

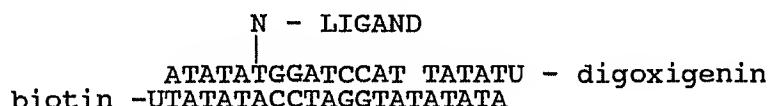
30 After an incubation period, the reaction mixture is diluted with an appropriate buffer and treated with restriction enzyme and then incubated with a streptavidin coated surface to immobilize the biotin molecules. Where a biological molecule with high affinity to the ligand on a 35 construct is present, the molecule will block access of the enzyme to the restriction site and prevent hydrolysis of the DNA scaffold, thereby resulting in the immobilization of the intact double-stranded oligonucleotide. In the alternative, where a biological molecule does not bind to the ligand on a construct, the restriction enzyme will not be blocked, allowing hydrolysis of the DNA scaffold as seen above, and resulting in the immobilization of a truncated construct,

free of the digoxigenin-labeled portion. A standard enzyme-linked immunosorbent assay (ELISA) with digoxigenin-peroxidase antibody can be used to detect the presence of digoxigenin on the streptavidin surface. A positive assay indicates that the restriction enzyme was blocked and this is

5 an indication that a molecule was bound to the linker. Thus, this approach can be used to detect the interaction between any ligand and any receptor having sufficient affinity and size to block access of a restriction enzyme to its restriction site.

10 In a modification of the assay described above, a "gap" (deletion of a base) is inserted in one of the strands of the double stranded sequence and a ligand(s) attached next to the gap as shown below:

15



20

(SEQ. ID ____)

Treatment of this construct with an endonuclease such as mung bean nuclease or nuclease S1 will result in hydrolysis of the construct at the gap region. Reaction of the hydrolyzed mixture with immobilized avidin or streptavidin

25 and subsequent washing will eliminate the digoxigenin containing sections of the construct. As described above, incubation of this construct with receptors having high affinity for the ligand prior to addition of a nuclease will prevent hydrolysis of the construct at the gap region and a

30 strong positive response will be obtained during an assay for the presence of digoxigenin.

In further variations of the DNA restriction site assay, the DNA scaffold can be replaced by a backbone comprised of peptides or peptoids or any polymer with a centrally located

35 bond that can be cleaved by a particular enzyme or other mechanism wherein cleaving can be blocked by occurrence of a ligand/receptor interaction near the site of the bond. For example, one could employ a commercially available poly(glycine) or poly(alanine) backbone into which has been inserted by standard techniques of peptide synthesis, a string containing a phenylalanine residue and a nearby ligand. An enzyme such as chymotrypsin A₁ (EC 3.4.21.1) can

then be used in analogous fashion to the nuclease described above. In addition, the assay could be modified to an SPA assay by using radioactive bases or ligands in the synthesis of the section of the construct opposite the biotin and attaching the streptavidin to scintillant loaded beads.

5 While any of the assays described herein are suitable for use in the present invention, this system provides several advantages such as, it does not require radioactivity, although radiolabelled nucleotides may be placed downstream of the restriction site to adapt the assay

10 to an SPA format; the system may be made ultra sensitive (detecting the presence of a single intact DNA backbone) by using PCR methodology for the detection system; the entire assay can be carried out in streptavidin coated microtiter dishes; and nucleotide analogs may be used to protect the DNA

15 backbone against nuclease activity in the sample, as long as the restriction site is not altered.

In this present invention, a membrane (available e.g., from Pall Corporation, East Hills, NY) containing particular reactive groups, may be placed into a well device, such as a

20 96-well dot blot apparatus. A library of compounds can then synthesize in the device, wherein a particular functional group added in the library synthesis couples with the particular reactive group on the membrane. For example, if the library compounds being synthesized contain an amino

25 group, the membrane will contain activate carboxylic acid groups and the groups will couple by amide bonding. Any excess groups on the membrane will be blocked with an appropriate blocking reagent. Then the ligand-bound membrane can be reacted with a biological sample that has previously

30 been derivatized, for example with biotin or radioactivity. An appropriate assay can then be used to detect ligand/receptor interactions.

5.3.4 EQUILIBRIUM PERTURBATION ASSAY

35 While the DNA restriction site assay described above may be characterized as an equilibrium perturbation assay, a preferable approach is to monitor the effect of a ligand/molecule interaction on the equilibrium of a DNA binding protein and its cognate sequence. For instance, the viral DNA binding protein UL9 may be used in a screen for compounds that bind to selected DNA test sequences that are placed downstream of the cognate UL9 sequence. The assay is

based upon the test sequences' ability to disturb the equilibrium of the binding protein that the binding protein does not bind to its cognate sequence. The assay as described in U.S. Patent No. 5,306,619, which is incorporated herein by reference.

5 In accordance with the principles of the present invention, the test sequence located downstream of the UL9 recognition site may serve as the backbone for the library of ligands, rather than as a target for DNA binding proteins as in U.S. Patent No. 5,306,619. A scaffold construct can be

10 formed that is comprised of the UL9 cognate sequence wherein a specific nucleotide base has been tagged with, for example, a marker such as biotin; and a ligand-derivatized oligonucleotide terminating in digoxigenin. It is known that the presence of the biotin on the cognate sequence does not

15 interfere with the binding of UL9 to the cognate sequence and, that once UL9 does become bound to the biotinylated sequence, the biotin molecule is blocked from immobilization, such as, on avidin or streptavidin. UL9 can be reacted with the construct and incubated until an equilibrium is

20 established between the UL9 and the biotinylated sequence. A biological sample can then be introduced into the reaction mixture whereby any receptors present in the sample that have affinity for the ligand on the scaffold test sequence will bind to the ligand, disturbing the equilibrium between the

25 binding protein and the biotinylated sequence, resulting in a change in the amount of free biotin available for binding with avidin or streptavidin.

In a variation of this assay method, a test sequence other than a second DNA sequence may be synthesized with the

30 screening sequence prior to attachment of the compounds. Any scaffold that allows for the attachment of the ligands may be used. Alternatively, the ligands may be covalently attached directly to the screening sequence without the need of an additional linker or scaffold.

35

6. EXAMPLES

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

**EXAMPLE 1: THE SCINTILLATION PROXIMITY ASSAY
IS A SENSITIVE METHOD FOR DETECTING THE
INTERACTION BETWEEN LIGANDS AND RECEPTORS**

The following example demonstrates the sensitivity of the scintillation proximity assay ("SPA"). This example 5 assesses the interaction between 2,5-diphenyloxazole (PPO)-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads and β -galactosidase using SPA.

E. coli transformed with a β -galactosidase expression vector were grown at 37°C in M9CA medium supplemented with or 10 without $H_2^{35}SO_4$ (0.1 mCi/mL of culture media). β -galactosidase expression was induced by treating the *E. coli* cultures with 1 mM IPTG for 3 hours. Subsequently, the *E. coli* cultures 15 were pelleted by centrifugation, resuspended in lysis buffer, and lysed by five freeze-thaw cycles. Bacterial debris was removed by centrifugation and the β -galactosidase was isolated from the supernatant using ammonium sulfate precipitation followed by affinity chromatography to yield 80% purification.

20 The interaction between unlabeled or labeled β -galactosidase and 2,5-diphenyloxazole (PPO)-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads (prepared by the method of Bertoglio-Matte in U.S. Patent 4,568,649) was assessed. The PPO-impregnated *p*-aminophenyl β -D- 25 thiogalactoside agarose beads were incubated overnight in a solution of 10% powdered milk in PBS (8.1 mMNa₂HPO₄, 1.5mM KH₂PO₄, 137mM NaCl, 2.7mM KCl, 0.5 mM MgCl₂, 0.9 mM CaCl₂) to block any sites responsible for non-specific binding. Alternatively, the beads may be blocked with agents such as 30 albumin, detergents, or even an extract from the control solution. Subsequently, 20 μ L of PPO-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads were mixed with 200 μ L of the ³⁵S- β -galactosidase (2.1×10^5 cpm when 35 counted in scintillation cocktail) along with either 200 μ L of PBS or 200 μ L of 1 mg/mL unlabeled β -galactosidase. In other experiments equal cpm ($6.5-7.7 \times 10^5$ cpm) of ³⁵S-labeled *E. coli* extracts or $H_2^{35}SO_4$ were mixed with 20 μ L of PPO-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads. The solutions were mixed for 10 minutes and then transferred to scintillation vials containing 1 mL of 0.2% casein in PBS. The interaction between the beads and the β -galactosidase or

$H_2^{35}SO_4$ was analyzed by assessing the radioactivity using a Beckman LS 6500 scintillation counter.

5 In the sample containing ^{35}S - β -galactosidase and PPO-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads, 487 +/- 52 counts per minute (cpm; n=3) were detected. In contrast, in the sample containing both labeled and unlabeled β -galactosidase and PPO-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads 341 +/- 16 cpm (n=3) were detected. These results demonstrate that unlabeled β -galactosidase can competitively displace the bead-associated, 10 labeled β -galactosidase. Furthermore, these results indicate that SPA can be used to detect mM interactions. Free aminophenylthiogalactoside was determined to have a Ki of only 1.9 mM. In addition, the results indicate that SPA can 15 be used to detect interactions between receptors and ligands with low affinity for each other. In this example, the beads affinity for β -galactosidase has been reduced approximately 2.3 fold by using PPO.

20 In the samples containing $6.5-7.7 \times 10^5$ cpm of ^{35}S -labeled *E. coli* extracts and PPO-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads, approximately 3000 cpm were detected. In contrast, only about 670 cpm were detected in samples containing 7.7×10^5 cpm of $H_2^{35}SO_4$ and PPO-impregnated *p*-aminophenyl β -D-thiogalactoside agarose beads. These 25 results indicate that the background due to the interaction between the beads and $H_2^{35}SO_4$ is very low. Therefore, the signal detected in the sample containing ^{35}S -labeled *E. coli* extracts is primarily due to the interaction between the beads and labeled *E. coli* protein. The high cpm detected for 30 the interaction between the beads and the ^{35}S -labeled *E. coli* extracts suggest that without the purification step, the background radioactivity would mask any specific β -galactosidase adsorption to the SPA beads.

35 This example demonstrates that the scintillation proximity assay (SPA) is a sensitive assay for detecting interaction between ligands and receptors at mM levels. Furthermore, the SPA is useful for detecting interactions between receptors and ligands with low affinity for one another.

EXAMPLE 2: THE DNA OBSTRUCTION ASSAY IS A SENSITIVE METHOD FOR ASSESSING THE INTERACTION BETWEEN RECEPTORS AND LIGANDS

The following example demonstrates the sensitivity of the DNA obstruction assay for assessing the interaction 5 between receptors and ligands.

The two oligomers shown below were synthesized by Clontech, Palo Alto, CA:

(SEQ. ID ____) I

10 5'BTT-TTT-TTT-TTT-TAT-ATA-GGA-TCC-TAT-ATA-TTT-TTT-TTT-TTT-
TTD-3'

B=biotin

D=digoxigenin

(SEQ. ID ____) II

15 5'AAA-AA-AAA-AAA-AAT-ATA-TAG-GAT-CCA-AAT-TAA-AAA-AAA-AAA-A-3'

The oligomers were designed such that a centrally located restriction site was formed upon annealing. The oligomers 20 were annealed using a 10:1 ratio of II:I by incubating the mixture in annealing buffer (10 mM Tris•Cl [pH 7.8] 0.1 M NaCl and 1.0 mM EDTA) at 65°C for 1 hour, then 57°C for 3 hours followed by storage at -20°C. Duplicate samples of 32.8 pmol of annealed oligomer were serially diluted in 25 annealing buffer and one of each duplicate dilution was hydrolyzed overnight with 40 units of restriction enzyme at 37°C as per instructions (New England BioLabs, Beverly, MA).

The remaining dilutions were treated the same except that restriction enzyme was omitted from the incubation mixtures.

30 Following an overnight incubation, the samples were added to streptavidin coated microtiter dishes and assayed using HRP derivatized anti-digoxigenin antibody and ABTS substrate as per instructions (Boehringer Mannheim GmbH, Mannheim, GER). Absorbances of the samples were then read at 405 nm.

35 Enzyme treated samples exhibited no absorbance over background (no oligomer in the incubation mixture), whereas the untreated samples exhibited clearly detectable absorbance at oligomer concentrations as low as 3 pmol. Thus, this system allows the detection of pmol levels of substrate with essentially no background.

EXAMPLE 3: THE ABILITY OF THE RESTRICTION ENZYME TO CLEAVE THE DNA SCAFFOLD IS AFFECTED BY THE MOLECULAR INTERACTIONS OF OTHER AGENTS WITH THE SCAFFOLD

This example illustrates that a molecular interaction
5 (ligand/receptor interaction) at a distance from a restriction site influences restriction enzyme activity.

To immobilize the DNA oligomers to the walls of the wells of a 96-well microtiter plate, 65.6 pmol samples of annealed DNA, labeled at one end with biotin and on the other with digoxigenin, and containing a centrally located restriction site, were added to streptavidin coated wells. A control well without DNA was also established (Table 1, lane 1). Four of the wells were then treated with anti-digoxigenin-peroxidase (anti-dig-POD) antibody in conjugate buffer (Boehringer Mannheim). Three wells were treated with conjugate buffer without antibody. After a 1 hour incubation, the wells were washed, and three of the wells were treated with 120 units of restriction enzyme for 1 hour at 37°C (Table 1, lanes 3, 5, and 7). The other wells were treated with the same solution without restriction enzyme. The wells were then washed again and assayed for intact (unhydrolyzed) oligomer by standard ELISA. The amount of complete, annealed DNA in the sample was indicated by the Vmax in mOD/mm.

25 The results for this experiment are compiled in Table 1 below. In the absence of DNA, the background Vmax value of the reaction was 0.89. In the absence of restriction enzyme and without pretreatment (Table 1, lane 2) the Vmax value of complete, unhydrolyzed annealed DNA was 30.96. In contrast, 30 the Vmax value was 2.52 when non-pretreated, immobilized DNA was treated with restriction enzyme (Table 1, lane 3). The decrease in the Vmax indicates that the restriction enzyme hydrolyzed the immobilized oligomer.

Pretreatment of the immobilized oligomer with anti-dig-
35 POD did not affect the assay (Table 1, lanes 4, 6, and 8) for intact oligomer since values of 34.96, 45.33 and 53.71, respectively, were obtained. However, pretreatment with anti-dig-POD did affect restriction enzyme activity (Table 1, lanes 5 and 7) because less hydrolysis of the oligomer occurred when the oligomer was pretreated prior to addition of the restriction enzyme. Pretreated samples that were later subjected to restriction enzyme (Table 1, lanes 5 and 7) had Vmax values of 15.17 and 14.64, respectively, whereas

oligomer that was not pretreated, but was restricted, had a Vmax of only 2.52. Thus, pretreatment of the oligomer with anti-dig-POD prior to the addition of the restriction enzyme reduced the activity of the restriction enzyme 17 fold, indicating that the interaction between anti-dig-POD and the 5 digoxigenin labeled DNA oligomer affected the ability of the restriction enzyme to cleave the oligomer. These results demonstrate that the addition of agents that interact with the oligomer to the reaction mixture affects the ability of the restriction enzyme to cleave the oligomer.

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Table 1				
Lane	Oligomer	Pretreatment with antibody	Restriction Enzyme	anti-digoxigenin-peroxidase (Vmax in mOD/min)
15	1	-	-	0.89
	2	+	-	30.96
	3	+	+	2.52
	4	+	-	34.96
20	5	+	+	15.17
	6	+	-	45.33
	7	+	+	14.64
	8	+	-	53.71

This example demonstrates that a "receptor" interacting with a ligand attached to a DNA backbone can strongly 25 influence restriction of the DNA backbone.

Examples 4-8 demonstrate the sensitivity of the fluorescence polarization assay and the differential binding pattern of biological samples to various probes. The 30 molecular probes used in these examples are listed in Table II.

EXAMPLE 4: THE FLUORESCENCE POLARIZATION ASSAY IS A SENSITIVE METHOD FOR DETECTING AN INTERACTION BETWEEN A PROBE AND A BIOLOGICAL SAMPLE

The following example demonstrates that the ability of various probes to bind to a component(s) of a biological sample varies with the concentration of the component(s) in the sample. Furthermore, this example demonstrates the differential binding of probes to a component(s) of a biological sample.

Serial dilutions of pooled human serum (Sigma Lot #116H4661) in PBS (pH=7.4) were prepared such that the concentrations of total protein in the samples were 65.6 mg/mL, 13.1 mg/mL, 2.6 mg/mL, 0.52 mg/mL, and 0.1 mg/mL. Then, 150 μ L of PBS and 1.0 μ L of a DMSO solution containing 5 probe #5 (Table II) at a concentration of 1.0×10^{-3} mg/mL were added to wells A1-A6 of a 96-well microtiter plate. Subsequently, 10 μ L samples of human serum solution containing 65.6 mg/mL, 13.1 mg/mL, 2.6 mg/mL, 0.52 mg/mL, and 0.1 mg/mL of total protein were added to wells A1, A2, A3, 10 A4, and A5, respectively. No human serum solution was added to well A6. Wells B1-B6 were prepared as above to serve as a duplicate for the samples tested in wells A1-A6.

Similar to the description above, 150 μ L of PBS and 1.0 μ L of a DMSO solution containing probe #18 (Table II) at a 15 concentration of 1.0×10^{-3} mg/mL were added to wells C1-C6. Then 10 μ L samples of the human serum solution containing 65.6 mg/mL, 13.1 mg/mL, 2.6 mg/mL, 0.52 mg/mL, and 0.1 mg/mL of total protein were added to wells C1, C2, C3, C4, and C5, respectively. No human serum solution was added to well C6. 20 Wells D1-D6 were prepared as above to serve as a duplicate for the samples tested in wells C1-C6.

Probes # 20-24 (Table II) were similarly treated as described above. The 96-well microtiter plates were then read in a Fluorolite FPM-2 fluorescence polarization 25 microtiter system, and the resulting polarization (mP) data was averaged for duplicate runs and plotted against total protein. The results as depicted in Figure 1 demonstrate that the binding of the various probes to a component(s) of human serum varies considerably. Furthermore, the 30 concentration of the component(s) present in the human serum affects mP values (a measure of probe binding).

EXAMPLE 5: DOSE RESPONSE OF PROBE #25 TO
POOLED HUMAN SERUM AND TO
STREPTAVIDIN-HRP CONJUGATE

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This example demonstrates that probes may be used to differentiate between biological samples. Furthermore, this example demonstrates that the ability of a probe to bind to a component(s) of a biological samples varies as the concentration of the component varies. To determine whether a strong binding interaction to a probe could be observed in the presence of serum proteins, the dose response of probe

#25 (Table II) to pooled human serum and to streptavidin-HRP conjugate was determined. Serial dilutions of pooled human serum (Sigma Lot # 116H4661) in PBS (pH=7.4) were prepared such that the concentrations of total protein in the samples were 65.6 mg/mL, 13.1 mg/mL, 2.6 mg/mL, 0.52 mg/mL, and 0.1 mg/mL. Then, 150 μ L of PBS and 1.0 μ L of a DMSO solution containing probe #25 at a concentration of 1.0×10^{-3} mg/mL were added to wells A1-A6 of a 96-well microtiter plate. Subsequently, 10 μ L samples of the human serum solution containing 65.6 mg/mL, 13.1 mg/mL, 2.6 mg/mL, 0.52 mg/mL, and 0.1 mg/mL of total protein were added to wells A1, A2, A3, A4, and A5, respectively. No human serum solution was added to well A6. Wells B1-B6 and C1-C6 were prepared as above to serve as duplicates for the samples tested in wells A1-A6.

Serial dilutions of streptavidin-HRP conjugate (Sigma 59420) in PBS (pH=7.4) were prepared such that the concentrations of total protein in the samples were 1.0 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL, 0.0001 mg/mL, and 0.00001 mg/mL. Then, 150 μ L of PBS and 1.0 μ L of a DMSO solution containing probe #25 at a concentration of 1.0×10^{-3} mg/mL was added to wells A1-A7 of another 96-well microtiter plate. Subsequently, 10 μ L samples of human serum solution containing 1.0 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL, 0.0001 mg/mL, and 0.00001 mg/mL of total protein were added to wells A1-A6. No human serum solution was added to well A7. Wells B1-B7 were prepared as above to serve as a duplicate for the samples tested in wells A1-A7. The fluorescence polarization of the samples in both 96-well microtiter plates were then determined or measured with a Fluorolite FPM-2 fluorescence polarization microtiter system. The resulting polarization (mP) data was averaged for duplicate runs and plotted against the log of total protein.

The data as shown in Figure 2 illustrates that the binding of probe #25 to serum protein is quite weak and, under the conditions of the experiment requires approximately 35 100 μ g of serum protein before binding to the probe is observed. In contrast, binding of probe #25 to the streptavidin-HRP conjugate was complete in the presence of 1.0 μ g of streptavidin-HRP conjugate. Thus, probe #25 has a higher affinity for the streptavidin-HRP conjugate than for human serum.

EXAMPLE 6: DETECTION OF STREPTAVIDIN-HRP CONJUGATE USING PROBE #25 IN POOLED HUMAN AND BOVINE SERUM

The following experiment demonstrates that a component with high affinity for a probe in a mixed biological sample
5 can be detected at low concentrations.

To determine whether a small amount of a strongly binding protein (streptavidin-HRP conjugate) could be detected in the presence of serum proteins, pooled human serum was spiked with streptavidin-HRP conjugate and the
10 response to probe #25 (Table II) was determined. First, 150 μ L of PBS and 10 μ L of a DMSO solution containing probe #25 at a concentration of 1.0×10^{-3} mg/mL were added to wells A1-A3 of a 96-well microtiter plate. Then, 10 μ L samples of a human serum solution at a concentration of 13.1 mg/mL of
15 total protein (obtained by diluting Sigma pooled serum Lot # 116H4661 1:5 in PBS) were added to all three wells. Thus, the total amount of human serum in each well was 131 μ g. The three wells represent triplicate determinations for binding of 131 μ g of serum proteins to probe #25.

20 Next, 150 μ L of PBS and 1.0 μ L of a DMSO solution containing probe #25 at a concentration of 1.0×10^{-3} mg/mL were added to wells B1-B3 of a 96-well microtiter plate. Then, 10 μ L samples of a human serum solution containing 13.1 mg/mL of total protein (obtained by diluting Sigma pooled
25 serum Lot # 116H4661 1:5 in PBS) and 10 μ L samples of a streptavidin-HRP conjugate solution containing 0.1 mg/mL of total protein were added to wells B1-B3. The total amount of human serum protein in each well was 131 μ g, and the total amount of streptavidin-HRP conjugate protein was 1.0 μ g.
30 Wells B1, B2, and B3 represent triplicate determinations for binding of probe #25 to 1.0 μ g of streptavidin-HRP conjugate protein in the presence of 131 μ g of serum proteins.

The fluorescence polarization for samples in the microtiter plate was read in a Fluorolite FPM-2 fluorescence
35 polarization microtiter system. The resulting polarization (mP) data were averaged for the three determinations and a bar graph was constructed to show the change in polarization (mP) that occurred in the presence of the streptavidin-HRP conjugate and serum proteins as compared to the serum proteins alone. The results as shown in Figure 3 illustrate that the binding of the probe #25 to 1.0 μ g of streptavidin-HRP conjugate protein is easily detected in the presence of

131 μ g of serum protein. These results indicate that the fluorescence polarization assay is sensitive method for detecting the binding between a probe and a component(s) of a sample present at μ g/mL concentrations.

5 **EXAMPLE 7: CONSTRUCTION OF A SERUM "FINGERPRINT"
WITH PROBES #1-25 BOTH WITH AND
WITHOUT STREPTAVIDIN-HRP CONJUGATE**

The following experiment demonstrates that the presence of a small amount of a strongly binding component can alter 10 the fingerprint of a biological sample.

First, 150 μ L of PBS and 1.0 μ L of a DMSO solution of probes 1-12, each probe at a concentration of 1×10^{-3} mg/mL, were added to wells A1-A12 of 96-well microtiter plate. This resulted in wells A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, 15 A11, and A12 containing 1.0 μ L of a 1×10^{-3} mg/mL solution of probe #1, #2, #3, #4, #5, #6, #7, #8, #9, #10, #11, and #12 (Table II), respectively, as well as 150 μ L of PBS. Wells B1-B12 were treated in the same manner to serve as a duplicate for probes 1-12.

20 Next, 150 μ L of PBS and 1.0 μ L of a DMSO solution of probes 13-24, each probe at a concentration of 1×10^{-3} mg/mL, were added to wells C1-C12 of the same 96-well microtiter plate. This resulted in wells C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, and C12 containing 1.0 μ L of a 1×10^{-3} mg/mL solution of probe #13, #14, #15, #16, #17, #18, #19, 25 #20, #21, #22, #23, and #24 (Table II), respectively, as well as 150 μ L of PBS. Wells D1-D12 were treated in the same manner to serve as a duplicate for probes C13-C24. Then, 150 μ L of PBS plus 1.0 μ L of a 1×10^{-3} mg/mL DMSO solution of 30 probe #25 were added to wells E1 and E2. E1 and E2 served as duplicates for probe #25. Subsequently, 10 μ L of a 1:10 dilution of pooled human serum (Sigma Lot #116H4661) was added to wells A1-A12, B1-B12, C1-C12, D1-D12, and E1-E2 in the 96-well plate. The total protein content in the 10 μ L 35 serum aliquot was 65.6 μ g.

The fluorescence polarization of each well of the 96-well plate was then read in a Fluorolite FPM-2 fluorescence polarization microtiter system and the resulting polarization (mP) values were plotted in a bar graph to show a "fingerprint" of the serum sample. The results are shown in Figure 4.

Next, each well was spiked with 10 μ L of a streptavidin-HRP conjugate solution containing 0.1 mg/mL of total protein. The total streptavidin-HRP conjugate protein added to each well was 1.0 μ g. Afterwards, the plate was read in a Fluorolite FPM-2 fluorescence polarization microtiter system, 5 and the resulting polarization (mP) values were plotted in the bar graph shown in Figure 5. This figure depicts a "fingerprint" of the serum sample with added streptavidin-HRP conjugate. The results indicate that the presence of a small amount of streptavidin-HRP conjugate protein is detectable in 10 the serum samples and that it alters the fingerprint observed for serum. These results suggest the potential for using the fluorescence polarization assay to screen for probes which can differentiate between normal and abnormal components of a biological sample.

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Examples 8-9 demonstrate that the fluorescence polarization assay can be utilized to screen libraries of probes for those that distinguish complex mixtures from each other.

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EXAMPLE 8: THE IDENTIFICATION OF PROBES WHICH DIFFERENTIALLY BIND TO HUMAN AND BOVINE SERA

The interaction between the 4,4-difluoro-5,7-dimethyl-4-25 bora-3a, 4a-diaza-s-indacene-3-proponic acid (BO)-labeled compounds 1-19 (Molecular Probes, Eugene, OR) listed in Table II and fetal calf serum or human sera was determined by fluorescence polarization. Because the amount of protein in a sample effects binding to a probe, the total amount of 30 protein in the human and fetal bovine sera for this experiment was determined. The human serum sample (Sigma Lot #116H4661) contained 65.6 mg/mL of total protein while the fetal bovine serum (Sigma Lot #96H4615) contained in 47.3 mg/mL of total protein. To minimize effects due to total 35 protein concentration, the experiment was run using 10 μ L portions of fetal bovine serum and $47.3/65.6 \times 10\mu\text{L} = 7.2\mu\text{L}$ of human serum.

To wells A1-A10, B1-B10, C1-C9, D1-D9, E1-E10, F1-F10, G1-G9, and H1-H9 of a 96-well microtiter plate 150 μ L of PBS was added. To wells A1-A10, B1-B10, C1-C9, D1-D9, E1-E10, F1-F10, 1.0 μ L of dimethyl sulfoxide (DMSO) solution of compounds 1-10, each compound at a concentration of 1×10^{-3}

mg/ml was added. In this manner, wells A1, B1, E1 and F1 each contained 150 μ L of PBS plus 1.0 μ L of a 1 X 10⁻³ mg/ml DMSO solution of compound #1. Similarly, wells A2, B2, E2 and F2 contained 150 μ L of PBS plus 1.0 μ L of a 1 X 10⁻³ mg/ml DMSO solution of compound #2 and so on for wells 3-10. To 5 wells C1-C9, D1-D9, G1-G9 AND H1-H9, 1.0 μ L of a DMSO solution of compounds 11-19, each compound at a concentration of 1 X 10⁻³ mg/ml was added. In this matter, wells, C1, D1, G1 and H1 each contained 150 μ L of PBS plus 1.0 μ L of a 1 X 10⁻³ mg/ml of PBS plus 1.0 μ L of a 1 X 10⁻³ mg/ml DMSO solution of 10 compound #12 and so on for wells 13-19. The plate was then read in a Fluorolite FPM-2 fluorescence polarization microtiter system with a 485 nm excitation filter and a 530 nm emission filter. The resulting polarization (mP) and intensity data represents a blank reading.

15 Next, to wells A1-A10, B1-B10, C1-C9, D1-D9, which contained PBS and a probe as described above, 7.2 μ L of human serum (Sigma Lot #116H4661) was added. Thus, series A1-A10 and B1-B10 were duplicates for compounds 1-10 and series C1-C9 and D1-D9 were duplicates for compounds 11-19 in human 20 serum. Similarly, to wells E1-E10, F1-F10, G1 -G19, and H1-H9, which contained PBS and a probe as described above, 10 μ L of fetal bovine serum (Sigma Lot #96H4615) was added. Thus, series E1-E10 and F1-F10 are duplicates for compounds 1-10 and series G1-G9 and H1-H9 were duplicates for compounds 11-25 19 in fetal bovine serum. The plate was then read in a Fluorolite FPM-2 fluorescence polarization microtiter system as described above. The resulting polarization (mP) and intensity data is depicted in Figure 6. The mP value for each probe is plotted as a bar graph. The first two bars 30 represent the values for the human serum duplicates and the last two bars represent the values for the bovine serum duplicates for each probe. This mP data represents the extent of polarization from the tag emission. A high reading indicates that the tagged compound was bound to a 35 macromolecule and a low reading indicates that the tagged compound was less bound to a lesser extent. As can be seen, compounds #2 and #18 are bound to a macromolecule to a greater extent in human serum than in fetal bovine serum.

EXAMPLE 9 : THE IDENTIFICATION OF PROBES WHICH DIFFERENTIALLY BIND TO BIOLOGICAL SAMPLES FROM DIFFERENT MAMMALIAN SPECIES

The following example demonstrates that biological samples can be distinguished from one another, including distinguishing species from species and diseased from undiseased, based on the differential binding pattern generated when they are screened with a library of probes.

1 μ L of each of the samples listed in Table 3 were combined with fluorescently tagged molecules in 96-well microtiter plates and the fluorescence polarization was measured using a Fluorolite FPM-2 fluorescence polarization microtiter system. The following fluorescently labeled probes were utilized in the fluorescence polarization assays: BODIPY labeled myo-inositol-1-phosphate (Probe 18; Molecular Probes) at about 0.18 nM final concentration, fluorescein labeled phenytoin (Probe 70; Sigma) at 10.5 nM final concentration, fluorescein labeled probe 129 (Sepracor) at 0.096 μ g/ml final concentration, fluorescein labeled probe 135 (Sepracor) at 0.078 μ g/ml final concentration, and fluorescein labeled probe 147 (Sepracor) at 0.09 μ g/ml final concentration. Fluorescence polarization values for the interaction between each probe and blood plasma or cell extracts were determined.

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Table 3. List of Biological Samples Used in the Assays.

Sample number	Source
1	Pooled human blood plasma
2	Pooled calf serum
3	Pooled fetal calf serum
4	Cynomologous monkey blood plasma
5	Baboon blood plasma
6	Rhesus monkey blood plasma
7	African green monkey blood plasma
8	Pooled Squirrel monkey blood plasma
9	Pooled WKY rat blood plasma
10	Pooled SHR rat blood plasma
11	Chicken embryo fibroblast extract
12	Src transformed chicken embryo fibroblast cell extract

Based upon the fluorescence polarization values, BODIPY labeled myo-inositol-1-phosphate (Probe 18) demonstrated differential binding for samples from different species (Figure 7). For example, the fluorescence polarization value for pooled human plasma was almost 3-fold higher than the 5 fluorescence polarization values for pooled calf serum or pooled fetal calf serum with Probe 18. However, little difference in the fluorescence polarization values was observed for samples from the same species. For example, no significant difference in the fluorescence polarization 10 values was detected for pooled WKY rat blood plasma and pooled SHR rat blood plasma with Probe 18. These results indicate that probes such as myo-inositol-1-phosphate can be used to obtain information about samples from different species.

15 The fluorescence polarization values for fluoresceinated phenytoin (Probe 70) indicate that it would also be a useful probe to differentiate the blood plasma from varies species (Figure 8). For example, the fluorescence polarization value obtained with Probe 70 for pooled human plasma was much 20 higher compared to the other samples. Additionally, a significant difference in the fluorescence polarization values was also detected between pooled calf serum and pooled fetal calf serum. These results suggest that probes such as fluoresceinated phenytoin can be useful in distinguishing not 25 only samples from different species but also samples from the same species at different times during development.

Probes with similar structure may also be useful in distinguishing samples from different species. Probes 147, 129, and 135, which are similar in structure (Figures 9A, 30 10A, and 11A), synthesized by solid phase chemistry and built upon a fluorescein backbone, provided sufficient information for the classification of blood samples from the six primates listed in Table 3. Probes 147 and 129 demonstrated higher polarization values after incubation with primate (human and 35 non-human) blood plasma than with non-primate blood plasma (Figures 9B and 10B). However, probe 129 exhibited a higher polarization signal when incubated with blood plasma from monkeys than with blood plasma from humans (Figure 10B). The fluorescence polarization value observed for probe 135 was higher when it was incubated with a sample of African green monkey plasma than with the plasma samples from other monkeys or humans (Figure 11B). Therefore, the differential binding

pattern of probes 129, 135, and 147 to primate blood plasma samples can be complied so that the information may be used to accurately distinguish the samples from one another.

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EXAMPLE 10: THE IDENTIFICATION OF PROBES
WHICH DISTINGUISH *STAPHYLOCOCCUS*
AUREUS FROM METHICILLIN RESISTANT
STAPHYLOCOCCUS AUREUS

5 The following experiments were performed to identify probes which could be used to distinguish *Staphylococcus aureus* (*S. aureus*), methicillin resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli* (*E. coli*).

10 *Staphylococcus aureus* (SA), methicillin resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli* (*E. coli*) were inoculated into 25 ml or 35ml volumes of Brain Heart Infusion medium (BHI, Difco). The cultures were incubated in a 37°C shaker until the O.D._{600nm} values were 0.344 for *E. coli*, 0.411 for SA and 0.367 for MRSA. Any differences in the O.D._{600nm} values of the cultures from those above are indicated in the tables. At this time 5 μ l samples of cultures were diluted into 100 μ l of phosphate buffered saline (PBS) + 0.1% bovine gamma globulin BGG in 96-well black, fluorescence polarization microtiter dishes. Subsequently, 15 5 μ l of diluted fluorescently labeled probes from the library having a final concentration of about 10nM were added to the cell suspensions and the plates were incubated for 30 minutes at 37°C or the indicated incubation period. Following the 20 incubation period, the amount of fluorescence polarization was determined for each sample using the Fluorolite FPM-2 25 fluorescence polarization microtiter system. Duplicate determinations of the fluorescence polarization were performed for each probe.

30 The fluorescently labeled probes used in the fluorescence polarization assays were purchased from outside sources or synthesized at Sepracor. Table 4 lists the probe name, the fluorescent label utilized, the source of the probes, and the probe number.

Table 4. Library of Fluorescently Tagged Probes.

Probe Number	Probe Name	Fluorescent Label	Source
5	2A or 1	BODIPY	Molecular Probes
	2B or 2	BODIPY	Molecular Probes
	2C or 3	BODIPY	Molecular Probes
	2D or 4	BODIPY	Molecular Probes
	2E or 5	BODIPY	Molecular Probes
10	2F or 6	BODIPY	Molecular Probes
	2G or 7	BODIPY	Molecular Probes
	2H or 8	BODIPY	Molecular Probes
	3A or 9	BODIPY	Molecular Probes
	3B or 10	BODIPY	Molecular Probes
15	3C or 11	BODIPY	Molecular Probes
	3D or 12	BODIPY	Molecular Probes
	3E or 13	BODIPY	Molecular Probes
	3F or 14	BODIPY	Molecular Probes
	3G or 15	BODIPY	Molecular Probes
20	3H or 16	BODIPY	Molecular Probes
	4A or 17	BODIPY	Molecular Probes
	4B or 18	BODIPY	Molecular Probes
	4C or 19	BODIPY	Molecular Probes
	4D or 20	Fluorescein	Molecular Probes
25	4E or 21	Fluorescein	Molecular Probes
	4F or 22	Fluorescein	Molecular Probes
	4G or 23	Fluorescein	Molecular Probes
	4H or 24	Fluorescein	Molecular Probes
	5A or 25	Fluorescein	Molecular Probes
30	5B or 26	BODIPY	Molecular Probes
	5C or 27	BODIPY	Molecular Probes
	5D or 28	Fluorescein	Molecular Probes
	5E or 29	Fluorescein	Molecular Probes
	5F or 30	Fluorescein	Sepracor
35	5G or 31	Fluorescein	Sepracor
	5H or 32	Fluorescein	Sepracor
	6A or 33	Fluorescein	Sepracor

Probe Number	Probe Name	Fluorescent Label	Source
6B or 34	perhexiline	Fluorescein	Sepracor
6C or 35	1-(4-	Fluorescein	Sepracor
5 6D or 36	Sep0119288	Fluorescein	Sepracor
6E or 37	serotonin	coumarin	Sepracor
6F or 38	773-99-2	coumarin	Sepracor
6G or 39	dehydroabietylamine	coumarin	Sepracor
10 6H or 40	norphenylephrine	coumarin	Sepracor
7A or 41	1-piperonylpiperazine	coumarin	Sepracor
7B or 42	4-(2-keto-1-benzimidaz...	coumarin	Sepracor
7C or 43	norfloxacin	coumarin	Sepracor
15 7D or 44	enoxacin	coumarin	Sepracor
7E or 45	1-(5-	coumarin	Sepracor
7F or 46	desferroxamine	fluorescein	Molecular Probes
7G or 47	form-nle-leu-phe-nle-tyr-	fluorescein	Molecular Probes
20 7H or 48	newport green	newport green	Molecular Probes
8A or 49	DHPE	fluorescein	Molecular Probes
8B or 50	Fluo-3AM cell permeant	dichlorofluorescei	Molecular Probes
8C or 51	SCH-23390	fluorescein	Molecular Probes
25 8D or 52	gly-gly-his	fluorescein	Molecular Probes
8E or 53	colchicine	fluorescein	Molecular Probes
8F or 54	5-dodecanoylamino	fluorescein	Molecular Probes
8G or 55	sodium green	sodium green	Molecular Probes
30 8H or 56	phen green	phen green	Molecular Probes
9A or 57	F-18	fluorescein	Molecular Probes
9B or 58	naltrexone	fluorescein	Molecular Probes
9C or 59	methotrexate	fluorescein	Molecular Probes
35 9D or 60	C8FDG	fluorescein	Molecular Probes
9E or 61	globotriose GB3	fluorescein	Molecular Probes
9F or 62	vancomycin	fluorescein	Sigma
9G or 63	total T4	fluorescein	Sigma
9H or 64	primadone	fluorescein	Sigma
10A or 65	amikacin	fluorescein	Sigma
10B or 66	theophylline	fluorescein	Sigma

Probe Number	Probe Name	Fluorescent Label	Source
5	10C or 67	fluorescein	Sigma
	10D or 68	fluorescein	Sigma
	10E or 69	fluorescein	Sigma
	10F or 70	fluorescein	Sigma
	10G or 71	fluorescein	Sigma
10	10H or 72	fluorescein	R&D System
	11A or 73	fluorescein	Sepracor
	11B or 74	fluorescein	Sepracor
	11C or 75	Tetanus toxin, C-fragment	Sepracor
	11D or 76	fluorescein	Sepracor
15	11E or 77	fluorescein	Sepracor
	11F or 78	fluorescein	Sepracor
	11G or 79	fluorescein	Sepracor
	11H or 80	fluorescein	Sepracor

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Based upon the fluorescence polarization values, four probes (11A, 10B, 6D, and 10E) showed differential binding to the Staphylococcal strains in comparison to BHI medium (not inoculated) or the *E. coli* sample (Table 5). The 25 fluorescence polarization assay was repeated using probes 10B, 10E, and 6D to determine if they could be used to distinguish SA from MRSA. Probes 10B and 10E did not demonstrate differential binding to *Staphylococcus aureus* (SA) in comparison to methicillin resistant *Staphylococcus aureus* (MRSA). No significant difference in the fluorescence 30 polarization values for SA, MRSA, *E. coli*, and uninoculated BHI medium samples were obtained with probes 10B or 10E (Table 6). Therefore, probes 10B and 10E are not useful for 35 differentiating SA from MRSA, *E. coli*, and uninoculated BHI medium.

Table 5. Fluorescence Polarization Values for the Bacterial Culture Samples Probed with Fluorescently Tagged Molecules.

Bact.	2A	3A	4A	5A	6A	7A	8A	9A	10A	11A	
5	E. coli	159.5 150.6	199.4 187.9	76.4 77.7	89.7 89.4	192.9 182.6	219.5 220.7	192.1 209.1	79.6 79.3	247.5 259.0	233.9 231.3
	SA	160.8 153.3	201.0 198.3	67.4 82.1	83.8 84.4	177.0 174.7	226.2 227.6	203.8 205.5	72.1 75.2	278.7 267.2	243.7 242.0
	MRSA	153.3 167.2	196.0 187.9	69.8 81.4	83.5 82.7	182.7 171.9	220.6 227.9	189.6 188.0	72.7 83.0	246.2 277.3	245.6 247.4
	BHI	153.4 148.6	191.2 186.8	80.3 76.4	85.3 80.3	192.1 174.9	224.0 218.5	206.7 217.1	75.8 73.8	259.8 271.5	240.8 248.1
10		2B	3B	4B	5B	6B	7B	8B	9B	10B	11B
	E. coli	193.0 187.6	32.6 28.1	145.2 154.0	93.0 94.1	76.7 78.6	176.0 174.2	236.9 254.7	41.3 45.4	56.2 52.2	130.0 103.3
	SA	193.1 178.7	38.8 25.4	154.7 146.3	89.3 81.1	75.0 85.2	173.0 169.7	239.2 243.3	37.4 39.7	65.1 61.5	134.3 104.8
	MRSA	197.0 176.0	29.0 25.2	146.7 154.2	87.9 77.8	73.7 66.2	173.6 174.9	252.4 232.6	42.8 43.8	60.7 53.0	130.5 112.8
15	BHI	189.4 210.9	34.5 31.7	156.4 160.7	83.8 92.1	72.9 82.8	169.9 174.2	249.0 262.1	38.8 38.9	51.7 55.7	134.8 101.8
		2C	3C	4C	5C	6C	7C	8C	9C	10C	11C
	E. coli	96.3 100.4	46.2 50.5	85.4 87.2	117.6 189.1	68.9 73.6	198.4 211.8	70.0 80.0	69.8 88.4	114.3 146.7	94.6 106.4
	SA	95.1 86.2	48.9 48.9	97.4 90.4	106.5 121.1	75.7 80.1	195.4 209.5	67.5 73.9	73.4 75.5	126.3 146.7	99.2 108.6
20	MRSA	98.7 92.1	44.4 46.7	90.4 86.7	122.1 110.0	71.2 74.1	122.1 110.0	71.2 74.1	74.2 78.1	135.2 128.9	113.4 111.4
	BHI	99.6 100.5	46.7 54.9	88.4 88.7	120.1 131.4	74.6 84.6	208.0 209.3	74.0 78.9	73.3 76.7	126.5 139.1	100.3 107.9
		2D	3D	4D	5D	6D	7D	8D	9D	10D	11D
	E. coli	161.6 178.1	56.0 49.9	54.2 51.5	50.6 46.8	261.7 272.5	209.9 223.0	134.6 130.4	63.5 68.2	177.5 198.4	111.1 113.6
25	SA	172.9 178.7	52.9 46.2	55.5 49.0	52.4 46.4	293.0 294.5	220.3 226.6	147.3 139.2	62.1 61.0	205.8 199.3	117.7 117.8
	MRSA	155.0 174.0	51.5 47.3	56.2 52.4	51.1 50.9	299.2 300.0	219.0 225.5	138.6 133.9	53.9 64.0	200.2 210.9	118.4 115.6
	BHI	164.6 194.0	50.0 48.6	54.7 50.0	47.8 42.6	271.8 273.1	217.9 223.0	154.7 136.7	57.6 67.4	203.1 213.5	102.6 119.1
		2E	3E	4E	5E	6E	7E	8E	9E	10E	11E
30	E. coli	91.7 92.4	59.4 45.8	120.2 122.2	57.6 53.2	238.7 230.7	229.6 230.8	53.0 52.6	54.1 51.0	307.0 310.2	132.3 122.8
	SA	99.8 104.0	59.5 54.1	141.4 136.8	59.3 58.1	251.2 245.3	231.6 241.7	56.5 55.2	55.9 47.2	328.3 327.8	133.8 123.5
	MRSA	93.6 99.3	58.6 52.3	131.4 129.1	59.7 53.7	246.4 231.8	225.6 233.4	53.2 50.7	50.5 47.2	320.4 325.6	138.3 126.4
	BHI	96.0 97.4	58.1 50.4	140.6 130.5	55.5 56.6	241.7 245.1	229.7 232.1	55.4 60.7	44.5 48.1	327.0 324.3	120.6 126.3
35											

	2F	3F	4F	5F	6F	7F	8F	9F	10F	11F	
5	E. coli	999.0 999.0	47.4 49.2	75.8 78.4	68.1 67.6	263.0 276.6	244.5 238.7	253.3 244.8	214.3 192.3	112.8 107.6	113.4 118.5
	SA	999.0 173.5	46.3 49.9	75.1 72.6	70.7 63.8	263.5 273.9	228.7 227.8	247.8 253.6	201.3 180.4	123.5 128.7	108.7 127.5
	MRSA	175.4 181.0	53.2 48.5	76.1 77.4	70.4 68.4	255.2 283.2	234.2 224.8	254.2 255.9	204.1 188.5	115.5 117.6	111.7 118.4
	BHI	158.0 181.1	54.2 46.6	778.4 78.2	66.4 68.3	251.8 282.9	228.8 246.2	234.1 264.4	191.0 195.9	110.4 121.6	97.6 122.0
10		2G	3G	4G	5G	6G	7G	8G	9G	10G	11G
	E. coli	272.2 270.6	46.8 49.8	78.1 80.3	188.9 204.5	230.2 205.2	143.8 134.8	37.2 38.7	38.4 34.0	74.2 75.1	43.6 37.3
	SA	261.5 270.8	43.5 37.9	79.0 82.9	201.6 223.7	236.0 227.9	129.5 140.0	41.5 44.5	29.1 41.7	73.8 80.8	44.4 50.4
	MRSA	262.3 255.5	43.0 37.	73.2 85.1	213.7 221.1	227.7 233.0	126.9 147.2	36.2 38.9	32.3 37.4	75.6 79.5	39.8 45.2
15	BHI	263.5 275.3	42.6 40.8	71.9 80.8	221.1 227.0	224.7 246.6	134.8 153.3	35.4 37.0	29.6 38.3	67.9 76.2	40.6 41.8
		2H	3H	4H	5H	6H	7H	8H	9H	10H	11H
	E. coli	108.6 96.5	75.6 71.1	279.9 311.8	174.2 190.7	245.7 270.0	259.4 278.7	55.5 51.8	99.2 96.4	149.4 158.1	67.7 73.3
	SA	104.7 95.4	90.2 70.4	308.7 308.9	213.1 230.5	270.0 281.8	250.2 266.9	57.4 57.2	98.3 93.4	161.0 175.2	63.1 72.2
20	MRSA	103.8 87.1	79.6 70.1	309.7 318.3	226.5 206.9	242.6 269.8	235.4 263.4	53.5 53.7	92.2 93.4	142.9 170.5	56.9 54.4
	BHI	94.2 93.7	85.2 76.0	297.7 303.6	212.4 231.5	254.8 269.3	257.2 283.8	55.3 63.9	93.2 97.3	159.3 162.5	55.8 67.8

Table 6. Probes 10B and 10E Are Not Useful In Distinguishing Bacterial Strains.

	Organism	Probe	Incubation Period (Hr)	O.D. _{600nm} Prior to Dilution	Fluorescence Polarization (MP)
25	E. coli	B10	7.5	1.068	75.6, 70.4, 70.8, 76.0, 82.2, 79.0
	S. aureus	B10	7.5	1.062	75.9, 79.8, 80.4, 70.7, 78.8, 73.3
	MRSA	B10	7.5	1.156	73.5, 73.9, 75.4, 76.0, 87.7, 79.7
	Medium (BHI)	B10	7.5	0.001	68.0, 74.0, 72.9, 69.6, 61.4, 73.7
35	E. coli	E10	7.5	1.068	70.9, 72.2, 72.7, 79.2, 75.9, 79.7
	S. aureus	E10	7.5	1.062	65.2, 80.8, 70.4, 71.6, 70.2, 75.4
	MRSA	E10	7.5	1.156	67.7, 72.5, 71.0, 72.9, 68.3, 78.1
	Medium (BHI)	E10	7.5	0.001	66.2, 70.7, 64.9, 69.5, 62.5, 62.5

The results from the fluorescence polarization assay with probe 6D indicate that this probe is useful for differentiating SA from MRSA, *E. coli*, and uninoculated BHI medium (Table 7; Figure 12). A higher fluorescence polarization value was obtained for *Staphylococcus aureus* 5 (SA) compared to methicillin resistant *Staphylococcus aureus* (MRSA), uninoculated BHI medium, and *E. coli*. This difference in the fluorescence polarization values was only detected when the bacterial samples were obtained from 10 cultures incubated for greater than 5 hours (Table 7; Figure 12). SA samples only demonstrated a higher fluorescence polarization value compared to MRSA samples when the culture incubation period was 7.5 hours or 24 hours. The 15 fluorescence polarization values obtained for uninoculated BHI medium were lower than the fluorescence polarization values obtained for SA and MRSA only when the culture incubation period was 7.5 hours or 24 hours. In contrast, *E. coli* samples consistently exhibited the lowest fluorescence 20 polarization values. Thus, the ability of probe 6D to bind to a component(s) of the bacterial samples changed as the 25 incubation period of the bacterial cultures increased.

The level of fluorescence polarization obtained with probe 6D increased over time for SA samples, but decreased with the other samples. For example, the fluorescence 30 polarization value for *E. coli* decreased from 211.8 +/- 8.8 mP for samples obtained from cultures incubated for 1 hour to 173.4 +/- 3.1 mP for samples obtained from cultures incubated for 24 hours (Table 7). In contrast, the fluorescence polarization value of SA increased from 223.3 +/- 5.1 mP for 35 samples obtained from cultures incubated 1 hour to 279.0 +/- 3.3 mP for samples obtained from cultures incubated 24 hours (Table 7). These results suggest that probe 6D preferentially binds to some unknown component(s) of SA and that the expression of this component(s) increases as the culture incubation period increases. Furthermore, the results indicate that probe 6D can be used to differentiate SA from MRSA.

This example demonstrates that this assay system is useful for screening for probes which differentiate bacterial strains. In this example one probe (6D) from a small library of 80 fluorescently labeled probes was found useful for

distinguishing the three strains of bacteria used in the experiments described above.

Table 7. Probe 6D is Useful for Distinguishing Between Bacterial Strains.

Organism	Incubation Period (Hr)	O.D. _{450nm} Prior to Dilution	Fluorescence Polarization (mP)
E. coli	1.0	0.004	211.8 (SD = 8.8)
S. aureus	1.0	0.018	223.3 (SD = 5.1)
MRSA	1.0	0.020	222.4 (SD = 4.4)
Medium (BHI)	1.0	0.002	219.8 (SD = 6.4)
E. coli	2.5	0.08	211.8 (SD = 3.8)
S. aureus	2.5	0.225	215.3 (SD = 3.9)
MRSA	2.5	0.157	219.2 (SD = 4.9)
Medium (BHI)	2.5	0.001	220.0 (SD = 4.0)
E. coli	5.0	1.068	207.8 (SD = 5.6)
S. aureus	5.0	1.062	231.9 (SD = 2.6)
MRSA	5.0	1.156	224.2 (SD = 6.7)
Medium (BHI)	5.0	0.001	227.9 (SD = 4.7)
E. coli	7.5	1.068	198.5 (SD = 7.9)
S. aureus	7.5	1.062	248.7 (SD = 1.4)
MRSA	7.5	1.156	234.9 (SD = 7.9)
Medium (BHI)	7.5	0.001	220.2 (SD = 5.2)
E. coli	24	1.643	173.4 (SD = 3.1)
S. aureus	24	1.781	279.0 (SD = 3.3)
MRSA	24	1.723	207.1 (SD = 3.4)
Medium (BHI)	24	0.002	202.7 (SD = 2.8)

EXAMPLE 11: THE ABILITY OF PROBE 6D TO DISTINGUISH STAPHYLOCOCCUS AUREUS FROM OTHER BACTERIAL STRAINS

The following experiments were performed to determine whether probe 6D could be used to distinguish *Staphylococcus aureus* (SA) from other bacterial strains.

Twelve cultures were inoculated into 35 ml volumes of Brain Heart Infusion medium (BHI, Difco) with five different bacterial strains. Although the identity of each culture was unknown to the experimenter, it was known that four of the

cultures contained *Staphylococcus aureus* (SA), two contained methicillin resistant *Staphylococcus aureus* (MRSA), two contained quinoline resistant *Staphylococcus aureus* (QRSA), and two contained vancomycin resistant *Enterococcus faecium* (VREF). The cultures were incubated overnight in a 37°C shaker. The next day seven, 5 µl samples were taken from each of the twelve cultures and diluted into 100 µl of phosphate buffered saline (PBS) + 0.1% BGG in 96-well black, fluorescence polarization microtiter dishes. Subsequently, 5 µl of approximately 10 nM probe 6D fluorescein tagged Sep0119288, was added to the cell suspensions and the plates were incubated for 30 minutes at 37°C or the indicated incubation period. Following the incubation period, the amount of fluorescence polarization was determined for each sample using the Fluorolite FPM-2 fluorescence polarization microtiter system. The high and low values of the polarization readings for the seven samples from each of the twelve unidentified cultures were discarded, and the remaining five values were averaged and the standard deviations determined.

The fluorescence polarization values determined for bacterial culture samples containing bacterial cells are shown in Table 8. The results from the fluorescence polarization assays for the twelve cultures were grouped based upon their fluorescence polarization values. These groupings are shown in Figure 13. Each grouping corresponded to a bacterial strain (Table 9). SA cultures had the highest fluorescence polarization values and the *E. coli* cultures had the lowest fluorescence polarization values (Figure 13). Therefore, with this single fluorescent probe twelve unknown cultures could be accurately grouped into five strains and SA could be distinguished from the other organisms used in the study.

Table 8. Fluorescent Polarization Values for the Twelve Unidentified Cultures Probed with 6D.

Unknown	MP	SD	N
1	221.4	1.140	5
2	237.6	3.578	5
3	210.0	2.739	5
4	233.8	3.114	5
5	154.0	4.062	5
6	245.8	2.775	5
7	208.8	1.789	5
8	216.6	2.608	5
9	227.2	1.924	5
10	235.4	1.517	5
11	214.8	4.438	5
12	153.2	1.304	5

Table 9. Grouping the Twelve Unidentified Bacterial Cultures into Five Groups Based on Fluorescence Polarization Values Corresponded to the Bacterial Strain.

Unknown	Organism
1, 9	QRSA
2,4,6,10	SA
3,7	MRSA
5,12	E. coli
8,11	VREF

This example demonstrates that the present invention provides a method of distinguishing between bacterial strains. Furthermore, that this example indicates that the technology of the present invention can be used to detect and diagnose infectious microorganisms.

EXAMPLE 12: SYNTHESIS OF LIGAND LIBRARIES

PROCEDURE 1: SOLUTION PHASE SYNTHESIS OF FLUORESCENTLY LABELED AMINES

Primary and secondary amines were treated individually with FITC (1.2 equivalents) in presence of dimethylaminopyridine (DMAP) in DMF or DMSO at ambient

temperature (if amine salts were used, one equivalents of triethylamine was added to free the amines). After approximately 24 hours, 2 equivalents of amine scavenger resin (tris-(2-aminoethyl)-amine polystyrene HL resin) were added to the reaction mixture. After 24-48 hours, the 5 mixtures were filtered to remove the resins and the filtrates containing the purified fluorescent-labeled ligands were further diluted with DMSO to the desired concentration for screenings. Table 10 lists the amines fluorescently labeled by solution phase synthesis following Scheme I.

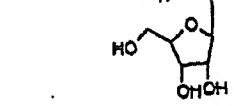
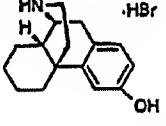
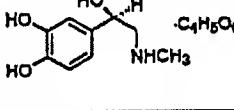
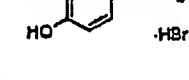
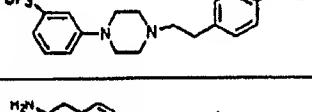
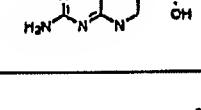
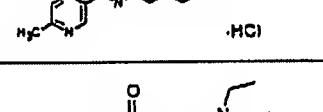
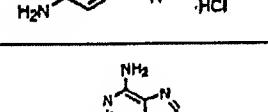
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Table 10. List of Amines Fluorescently Labeled

Reactions	Structure of amine (starting material)	MW (Cat. No.)	Mmol used	Weigh (mg)	HPLC purity of ligand (area % at 220nm)	
15	1		301.77 (R107)	0.01	3.02	89.1%
20	2		235.11 (R106)	0.01	2.35	82.0%
25	3		260.2 (D002)	0.01	2.60	86.8%
30	4		387.39 (S010)	0.01	3.87	92.1%
35	5		428.5 (X100)	0.01	4.29	84.73%
	6		189.6 (D019)	0.01	1.89	94.22%
	7		207.75 (C108)	0.01	2.08	90.38%

8		213.67 (B020)	0.01	2.14	85.12%
9		365.9 (A225)	0.01	3.66	78.41%
10		377.4 (W101)	0.01	3.77	93.32%
11		199.17 (W105)	0.01	1.99	74.80%
12		249.64 (P118)	0.01	2.50	80.71%
13		324.2 (H123)	0.01	3.24	96.48%
14		213.19 (B004)	0.01	2.13	83.34%
15		357.6 (D101)	0.01	3.58	94.43%
16		223.49 (B012)	0.01	2.23	87.25%

	16		226.56 (D103)	0.01	2.27	77.96%
5	17		356.23+ 1.5 H ₂ O (B116)	0.01	3.83	78.28%
10	18		448.54 (D146)	0.01	4.49	84.39(?)
15	19		313.79 (A129)	0.01	3.14	79.40%
20	20		228.7 (S008)	0.01	2.29	85.13%
25	21		202.3 (M008)	0.01	2.02	77.54%
30	22		211.69 (H101)	0.01	2.12	83.11%
35	23		364.29 (H139)	0.01	3.64	82.41%
	24		260.2 (D002)	0.01	2.60	92.62%
	25		232.27 (A122)	0.01	2.32	82.69%

5	26		386.41+ 0.5H ₂ O (A202)	0.01	3.95	89.10%
10	27		324.26+ 0.3H ₂ O (H156)	0.01	3.30	92.28%
15	28		333.30 (E104)	0.01	3.33	83.03%
20	29		220.07 (D106)	0.01	2.20	86.94%
25	30		349.4 (S009)	0.01	3.49	73.21%
30	31		266.34 (A139)	0.01	2.66	87.79%
35	32		237.22+ 1.3H ₂ O (S154)	0.01	2.71	76.17% (?)
40	33		329.79 (G119)	0.01	3.30	78.83%
45	34		271.79 (P137)	0.01	2.72	79.02%
50	35		308.29+ 0.75H ₂ O (A014)	0.01	3.23	81.14%

PROCEDURE 2: SYNTHESIS OF AMINE CARBAMATE WANG RESIN

Wang resin (25 g, 20 mmol, 0.8 mmol/g loading) and carbonyl diimidazole (CDI) (20 g, 120 mmol) in dry THF (180 ml) are shaken at room temperature for 2 days. The mixture is filtered and the resin washed thoroughly with DMF, THF and 5 CH_2Cl_2 and dried to give imidazole carbonyl Wang resin (27 g).

The imidazole carbonyl Wang resin (5 g, 4 mmol, 0.8 mmol/g loading) is then treated with piperazine (1.7 g, 20 mmol), homopiperazine (2.0 g, 20 mmol) or 4,4'-trimethylenedipiperidine (4.2 g, 20 mmol) in a separate 10 reaction tube in THF/DMF (1:1, 40 ml) at room temperature for 17 hours. The resin is then thoroughly washed with DMF, THF, and CH_2Cl_2 and dried under vacuum to give the corresponding amine carbamate Wang resin, respectively.

15 **PROCEDURE 3: SYNTHESIS OF DTAF ON WANG RESIN VIA AMINE CARBAMATE LINKER**

An amine carbamate Wang resin (1.0 equivalents) and DTAF hydrochloride (2.5 equivalents) in DMF in the presence of diisopropylethylamine (DIPEA) (4.0 equivalents) is shaken at 20 room temperature for 12-17 hours. The resin is filtered and washed thoroughly with DMF, THF and then CH_2Cl_2 and dried under vacuum to give monochlorotriazylfluorescein on Wang resin.

25 **PROCEDURE 4: SYNTHESIS OF DTAF ON TRITYL OR CHIORITYL RESIN VIA AMINE LINKERS**

An amino trityl or amino 2-chlorotriyl resin (Novabiochem) (0.12 mmol, 1.0 equivalents) and DTAF (0.3 mmol, 2.5 equivalents) in DMF (ml) in the presence of DIPEA 30 (0.48 mmol, 4.0 equivalents) is shaken at room temperature for 24-40 hours. The resin is washed with DMF, THF and then DCM and dried under vacuum to yield the monochlorotriazylfluorescein on trityl resin.

35 **PROCEDURE 5: SYNTHESIS OF DTAF ON RINK RESIN**

Rink amide resin (1 g, 0.8 mmol, 0.8 mmol/g loading) is treated with a 30% solution of piperidine in DMF (5 ml) for 2 hours and the resulting Rink amine resin is washed with DMF following DCM and dried. The Rink amine resin (0.8 mmol) is then shaken with DTAF (2.5 equivalents, 2 mmol) and DIPEA (4.0 equivalents, 3.2 mmol) in 5 ml of DMF for 24 hours. The resin is washed with DMF/THF and then DCM and dried under

vacuum to give the monochlorotriazylfluorescein on Rink resin.

Similarly, Fmoc-amino acid on Rink resin (prepared from Rink amine resin and Fmoc-amino acid under standard peptide synthesis conditions, e.g., DIC/DMAP/DCM) is treated with 5 piperidine in DMF to remove the Fmoc group. The amino acid Rink resin is then treated with 2.5 equivalents of DTAF and 4.0 equivalents of DIPEA in DMF for 24 hours at room temperature. After washing and drying, the monochlorotriazylfluorescein on Rink resin is obtained.

10

PROCEDURE 6: REACTION OF FLUORESCENT LINKER WITH A DIAMINE

Monochlorotriazylfluorescein on a solid support such as Wang resin or trityl resin is treated with 4.0 equivalents of 15 a symmetrical diamine in DMF at room temperature for 24-40 hours. The resin is then washed with DMF/MeOH followed by DCM and dried under vacuum to give an amino fluorescein on solid support.

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PROCEDURE 7: REPARATION OF A LIBRARY OF FLUORESCENT-LABELED UREA COMPOUNDS

Three amino acid Rink resin (i.e., L_1 of Scheme I is CH_3 , isobutyl, benzyl) (500 mg each, 0.4 mmol, 0.8 mmol/g loading) 25 are treated with DTAF (2.5 equivalents) as described above in Procedure 5, respectively, in separate tubes to give three different monochlorofluorescein derivatives on resins. These resins (250 mg each, 0.2 mmol) are then treated with piperazine (4.0 equivalents each) and 4,4'-trimethylenedipiperidine (4.0 equivalents each) individually 30 as in Procedure 6 to give six amino fluorescein labeled resins. The six amino fluorescein resins (25 mg, 0.02 mmol each) are then reacted with 10 isocyanates (3.0 eq each) in THF individually in a parallel fashion to give 60 35 fluorescent-labeled urea compounds on resins. The resins are then cleaved with 30% TFA/DCM followed by drying under vacuum to give 60 individually labeled fluorescent urea compounds (which are confirmed by HPLC/MS analysis) which are dissolved in 1 ml of DMSO for biological screening.

**PROCEDURE 8: ATTACHING DCSF ON WANG RESIN OR
TRITYL RESIN VIA AMINE LINKERS**

An amine carbamate Wang resin (4 mmol, 0.8 mmol/g) and DCSF (2.0 equivalents, 8 mmol) in 50 ml of dry DMF in the presence of DIPEA (2.0 equivalents, 8 mmol) is shaken at room 5 temperature for 2-3 days. The resin is then washed with DMF, MeOH followed by DCM and dried to give the monochlorosulfofluorescein on Wang resin.

In a similar manner, a piperazine trityl resin is treated with DCSF in the presence of DIPEA in DMF to give the 10 monochlorosulfofluorescein on trityl resin.

**PROCEDURE 9: REACTION OF MONOCHLOROSULFOFLUORESCEIN
WITH CYCLIC DIAMINE**

15 The fluorescein on Wang resin (1.33 mmol, 1.0 equivalents) from Procedure 8 is treated with a cyclic diamine such as piperazine (4.0 mmol, 3.0 equivalents) in 10 ml of DMF for 24 hours. The resin is then washed with DMF/MeOH followed by DCM and dried under vacuum to give amino 20 sulfofluorescein on Wang resin.

**PROCEDURE 10: PREPARATION OF
SULFOFLUORESCEIN LABELED LIBRARY**

The amino sulfofluorescein Wang resins containing 25 different diamine linkers (e.g., a combination of piperazine, homopiperazine and 4,4'-trimethylenedipiperidine) is treated in a parallel fashion with different organic acids (10 equivalents) in the presence of a coupling agent such as diisopropylcarbodiimide (DIC) (10 equivalents) and DMAP (5.0 30 equivalents) in DMF/DCM at room temperature for 24-48 hours. After the amine is completely acylated (followed by HPLC by cleavage of small amount of resin), the resin is washed with DMF/MeOH followed by DCM and dried under vacuum. The desired 35 fluorescent-labeled compounds are then obtained by cleavage from the resin using 30% TFA/DCM followed by vacuum drying.

**PROCEDURE 11: SYNTHESIS OF FLUORESCENT
LABELED QUINZALINONE**

In Step 1, Wang resin bound O-hydroxylamine (ref: Floyd, C.D., et al. *Tetrahedron Lett.* 1996, vol. 37, 8045) (2.0 g, 1.6 mmol, 0.8 mmol/g loading) is treated with isatoic anhydride (6.4mmol, 4.0 equivalent) in 25 ml of DMF in the

presence of DMAP (0.8 mmol, 0.5 equivalent) at 65-70°C with shaking for 26 hours. The resulting resin mixture is then filtered and washed thoroughly with DMF, MeOH and DCM and dried to give the N-hydroxyamide on resin.

In Step 2, the N-hydroxyamide resin 0.04 mmol, 50 mg, 5 0.8 mmol/g loading) is treated with commercially available first group of Fmoc-protected α -amino acid (0.2 mmol, 5.0 equivalent), coupling agent PyBrOP (Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate, 0.2 mmol, 5.0 equivalent) and DMAP (0.2 mmol, 5.0 equivalent) in dimethyl acetamide (DMAC) 10 solvent (0.6 ml) at 60-65°C with shaking for 20-24 hours. The resin is filtered and washed with DMF, MeOH and DCM thoroughly and dried to give the quinazolinone on resin. The resin is then suspended in 30% piperidine in DMF (0.6 ml) to remove the Fmoc-protecting group from the amine group to give 15 the free amino quinazolinone resin after filtration and washing for attachment of next reactive block.

In Step 3, the amino quinazolinone on resin (50 mg, 0.04 mmol) is treated with second group of Fmoc-protected amino acid (0.2 mmol, 5.0 equivalent), coupling agent DIC 20 (diisopropylcarbodiimide, 0.2 mmol, 5.0 equivalent), HOBr (N-hydroxybenzotriazole, 0.2 mmol, 5.0 equivalent) and DMAP (0.04 mmol, 1.0 equivalent) in DMF (0.6 ml) at room temperature for 20-24 hours. The resin is then filtered and washed thoroughly with DMF, MeOH and DCM and dried. The 25 resin is then suspended in 30% piperidine in DMF (0.6 ml) to remove the Fmoc-protecting group from the amine group to give the free amino resin after filtration and washing for attachment of the dye.

In Step 4, the amine resin (0.04 mmol) is treated with 30 DTAF•HCl dye (Dichlorotriazylamino fluorescein monohydrochloride, 0.08 mmol, 2.0 equivalent) and DIPEA (diisopropylethylamine, 0.16 mmol, 4.0 equivalents) in DMF (0.6 ml) at room temperature with shaking for 20-24 hours. The resin is then filtered and washed thoroughly with DMF, 35 MeOH and DCM and dried to give the fluorescent-labeled quinazolinone on resin.

In Step 5, the fluorescent-labeled resin (0.04 mmol) is treated with 30% Trifluoracetic acid (TFA) in DCM (1.0 ml) at room temperature with shaking for 1-2 hours. This mixture is filtered and the resin is rinsed with 0.5 ml of DCM. The combined filtrate is collected and evaporated under vacuum to

dryness to give the desired fluorescent-labeled quinazolinone ligand which may be dissolved in DMSO for screenings.

A library of the above fluorescent-labeled quinazolinone ligands are made by performing the above reactions in a parallel manner using different isatoic anhydrides and Fmoc-
5 protected amino acids.

PROCEDURE 12: SYNTHESIS OF LIGANDS USING UGI-COUPLING ON SOLID PHASE

Rink amine resin (1.0 equivalents) (Prepared by treatment
10 of commercial Rink amine resin with 25% piperidine in DMF) is swelled in MeOH/DCM (1:2, v/v). The aldehyde (10 equivalent) and Fmoc-protected amino acid (10 equivalent) are added. The mixture is shaken at room temperature for 1-2 hours. The isocyanide (10 equivalent) is then added. The mixture is
15 shaken at room temperature for 20-24 hours. The resin is then filtered and washed thoroughly with DMF, MeOH and DCM and dried. The resin is then treated with 25% piperidine in DMF to give the resin containing a free amine group. The resin is then treated with 2.0-3.0 equivalent of DTAF and 4-6
20 equivalents of DIPEA in DMF to give the fluorescent-labeled ligand on resin after filtration and washing as usual. The desired fluorescent-labeled ligand is then obtained after cleavage from the resin with TFA/DCM and drying.

25 EXAMPLE 13: THE USE OF FLUORESCENCE POLARIZATION TO DISTINGUISH AND IDENTIFY HUMAN BLOOD SERA SAMPLES

The following example illustrates the use of the present
30 invention to distinguish and/or identify two or more human blood sera samples. For example, normal sera and abnormal sera can be distinguished from each other based upon the differential binding pattern generated when the samples are screened with a library of probes. In addition, the
35 invention may be used, upon solution of the appropriate probes, to distinguish diabetic sera from normal or otherwise non-diabetic sera. The discussion below illustrates the general procedures, to be employed to distinguish between human sera samples.

Fluorescein labeled probes from three different library plates (XN1192-54, XN1043-58, GY1175-96 and Plate 1) are combined with sera samples purchased from Western States Plasma Company (Fallbrook, CA). The sera samples consisted

of diabetic sera pooled from three diabetic individuals (confirmed to be diabetic by reference laboratories) and pooled sera from normal individuals (Lot HS300; SeraCare, Oceanside, California).

First, 2.5 μ l of each fluorescein labeled probe (1-10 5 nM) was combined with 95 μ l of buffer (PBS + 0.03% lithium dodecysulfate) and 5 μ l of sera in 96-well plates. Then the mixtures were incubated at 37°C for 30 minutes. The sera samples, where indicated, were centrifuged at 20,000 xg for 3.5 hours and 5 μ l of the lower, straw colored sera layer was 10 assayed for probe binding. The fluorescence polarization for each probe with each sample was determined by utilizing the Fluorolite FPM-2 fluorescence polarization microtiter system. Each probe was tested with each sample in triplicate and the resulting fluorescence polarization values (MP) were 15 averaged.

In an initial screen with 80 fluorescein labeled probes, probes showed a significant difference in the fluorescence polarization values obtained for the two sera samples. These probes were screened again in triplicate under the same 20 conditions. The probes listed in Table 11 and depicted in Table 12 showed a significant difference in the fluorescence polarization values obtained for normal pooled sera (Lot HS300) and diabetic sera (Figure 14). The fluorescence polarization values obtained for 10 of the 12 probes listed 25 in Table 11 were higher for diabetic sera than for normal sera (Lot HS300). The results suggest that probes 58A2, 58A3, 58A6, 58B6, 58B7, 58B11, 58H8, 96G3, 54G3, and P1B4 are preferentially binding to some component(s) present in diabetic sera.

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Table 13 PROBES THAT DISTINGUISH SERA SAMPLES							
	Plate	Probe	Intensity Diabetic	MP Diabetic	Intensity Normal	MP Normal	Δ MP
5	XN1143-58	A2	146	326.8	123	301.5	25.3
XN1143-58	A3	209	241.7	195	216.4	25.3	
XN1143-58	A6	217	232.0	195	205.9	26.1	
10	XN1143-58	B6	217	233.6	199	203.3	30.3
XN1143-58	B7	245	209.9	225	182.8	27.1	
XN1143-58	B11	194	244.4	194	212.8	31.6	
XN1143-58	H8	166	277.4	146	247.5	29.9	
15	GY1175-96	G3	319	259.2	324	225.0	34.2
XN1192-54	C6	566	233.7	561	292.0	58.5	
XN1192-54	E8	314	250.0	313	297.3	47.3	
XN1192-54	G3	253	212.1	235	176.8	35.3	
20	PLATE 1	B4	395	181.3	372	159.7	21.6

One possible reason for the difference in the fluorescence polarization values obtained for the normal (Lot HS300) and diabetic sera samples is that the lipid content in diabetic sera is higher than in normal sera. In order to determine if the lipid content of the diabetic sera was affecting the fluorescence polarization values obtained, the sera samples were depleted of lipids by centrifugation and the fluorescence polarization was determined using the same probes listed in Table 11. The fluorescence polarization values obtained with the probes are listed in Table 12 and are depicted graphically in Figure 15. The magnitude of the difference between the fluorescence polarization values for diabetic sera and normal sera (Lot HS300) using probes 58A2, 58A3, 58A6, 58B6, 58B7, 58B11, 58H8, 96G3, 54G3, and P1B4 appears to be greater when the sera is depleted of lipids. The polarization values are higher for the lipid depleted diabetic sera than for undepleted diabetic sera. These results indicate that the lipid content of the diabetic sera does not explain the why diabetic sera compared to normal sera (Lot HS300) binds preferentially to probes 58A2, 58A3, 58A6, 58B6, 58B7, 58B11, 58H8, 96G3, 54G3, and P1B4.

Furthermore, these results suggest that a lipid component(s) present in the diabetic sera reduces the binding affinity of probes 58A2, 58A3, 58A6, 58B6, 58B7, 58B11, 58H8, 96G3, 54G3, and P1B4 for another component(s) present in diabetic sera.

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Table 14							
COMPARISON OF PROBE BINDING TO LIPID DEPLETED DIABETIC AND NORMAL HUMAN SERA							
	Plate	Probe	Intensity Diabetic	MP Diabetic	Intensity Normal	MP Normal	Δ MP
10	XN1054-58	A2	153	341.4	117	287.6	54.3
XN1043-58	A3	214	265.8	183	211.7	54.1	
XN1043-58	A6	222	251.9	202	209.6	42.3	
15	XN1043-58	B6	232	245.7	209	204.1	41.6
XN1043-58	B7	205	235.4	232	201	34.4	
XN1043-58	B11	205	260.6	197	226.2	34.4	
XN1043-58	H8	197	291.8	169	262.3	29.5	
20	GY1175-96	G3	343	268.2	376	229.8	22.5
XN1192-54	C6	593	247.4	622	269.9	22.5	
XN1192-54	E8	326	263.4	338	287.4	24.0	
XN1192-54	G3	273	208.9	270	208.9	0.0	
25	PLATE 1	B4	273	179.0	270	181	1.2

In the case of probes 54C6 and 54E8, the difference in the fluorescence polarization values between the lipid depleted diabetic sera and lipid depleted normal sera (Lot HS300) is relatively unchanged compared to the differences observed between lipid containing sera. However, the effect of removing the lipid content of the sera dramatically affects the difference in fluorescence polarization values obtained for normal (Lot HS300) and diabetic sera using probes 54G3 and P1B4. In the presence of lipids the fluorescence polarization values for the diabetic sera are significantly higher for these two probes than for the normal sera (Lot HS300). However, in lipid depleted sera the fluorescence polarization values for normal (Lot HS300) and diabetic sera using probes 54G3 and P1B4 are almost identical. These results suggest that probes 54G3 and P1B4 are binding to a lipid soluble ligand in the diabetic sera.

The effect of the lipid depletion on probe binding suggests that the probes are binding to a minimum of three different targets or more.

In summary, the results described above demonstrate that human sera samples can be distinguished from one another

5 using the fluorescence polarization-based assay described in the present invention. Furthermore, the methods utilized in this example can be used to identify probes that distinguish normal and diabetic sera.

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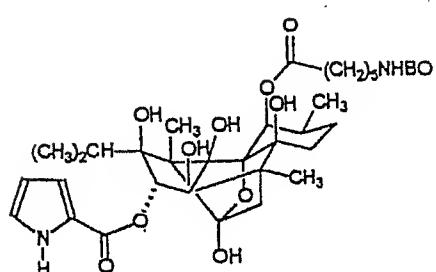
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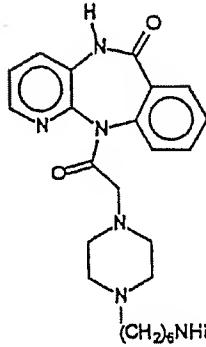
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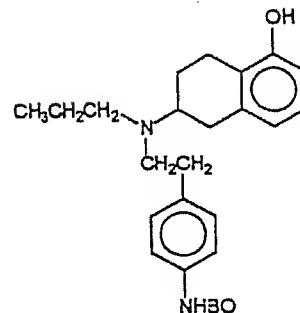
Table II
BO- and FL-Labeled Compounds for Examples 4-8



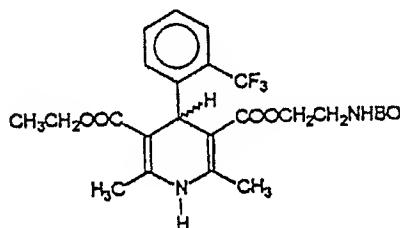
1. ryanodine



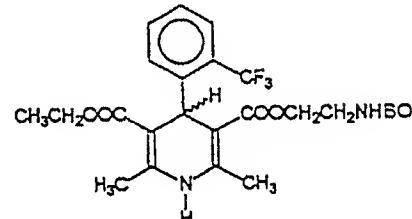
2. pirenzepine



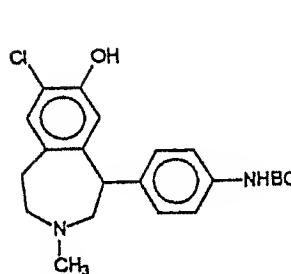
3. PPHT



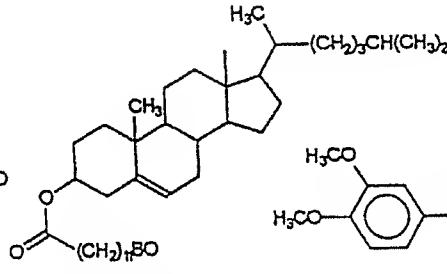
4. (+) dihydropyridine



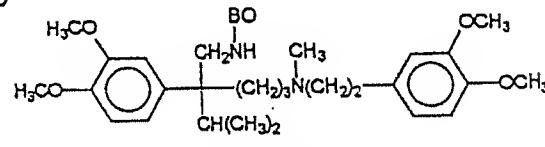
5. (-) dihydropyridine



6. SCH 23390

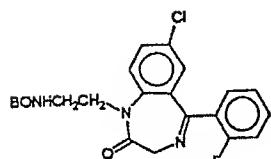


7. Cholesteryl C12

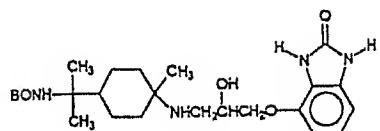


8. verapamil

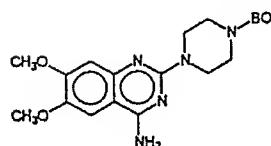
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BO- and FL-Labeled Compounds for Examples 4-8



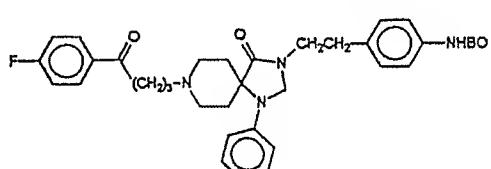
9. Ro1986



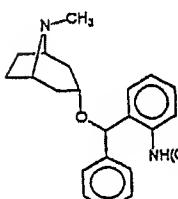
10. (+/-)CGP 12177



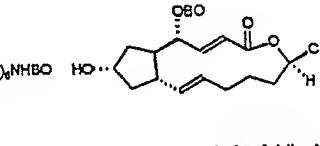
11. prazosin



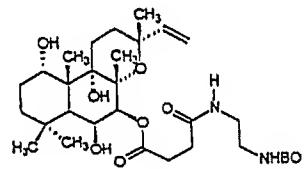
12. NAPS



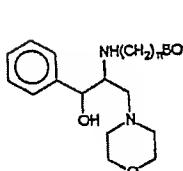
13. ABT



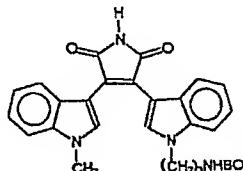
14. brefeldin A



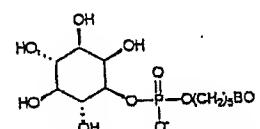
15. forskolin



16. C12-MPP

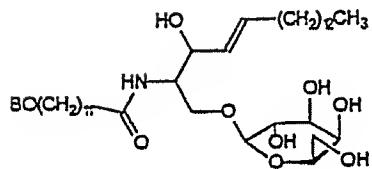


17. bis indolylmaleimide

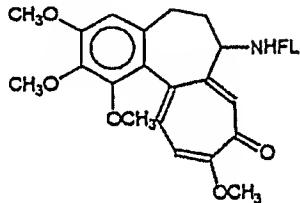


18. myo-inositol-1-phosphate

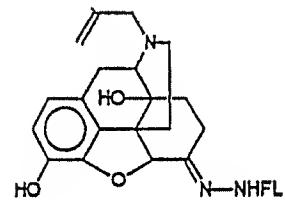
Table II (Continued)
BO- and FL-Labeled Compounds for Examples 4-8



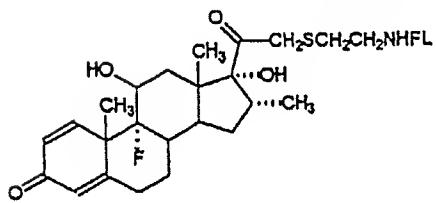
19. C12 galactocerebroside



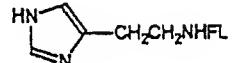
20. colchicine



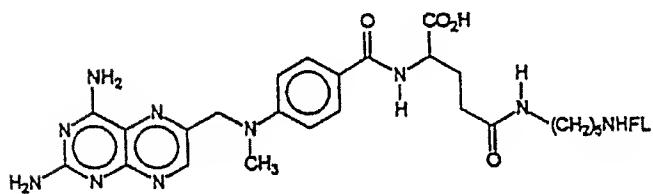
21. naloxone



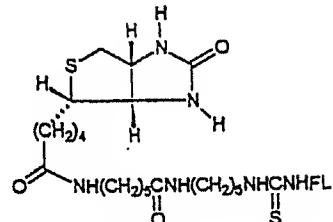
22. dexamethasone



23. histamine



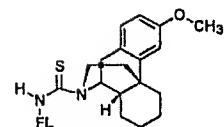
24. methotrexate



25. biotin

Table 12

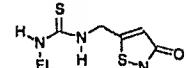
Structures for Probes Useful Within the Present Invention



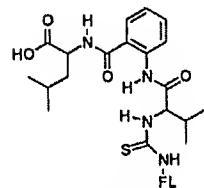
Probe 54C6



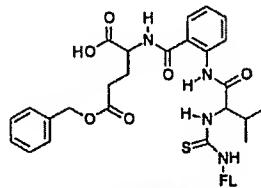
Probe 54E8



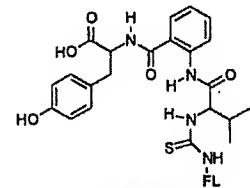
Probe 54G3



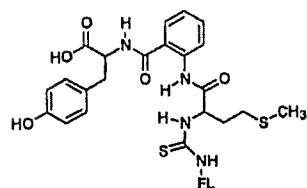
Probe 58A2



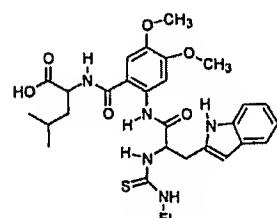
Probe 58A3



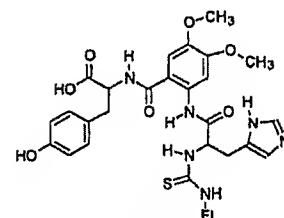
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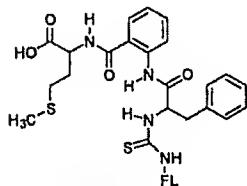
Probe 58B6



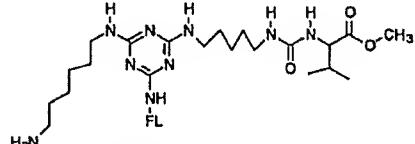
Probe 58B7



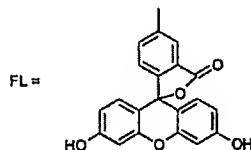
Probe 58B11



Probe 58H8



Probe 58G3



FL =

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention. Indeed, various modifications of the invention in addition to 5 those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are incorporated herein by 10 reference in the entirety for all purposes.

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WHAT IS CLAIMED IS:

1. A method for characterizing a sample composed of a mixture of molecular components, comprising:

5 - (a) contacting the sample with a library of molecular probes under conditions which allow the molecular probes to bind to their specific binding partners; and

10 (b) detecting any binding of individual members of the library of molecular probes to molecular components in the sample using a homogeneous assay system, in which the binding pattern of members of the library with the sample provides a molecular fingerprint of the sample.

15 2. The method of Claim 1 in which the members of the library of molecular probes are labeled.

20 3. The method of Claim 2 in which the label is a fluor.

25 4. The method of Claim 3 in which the binding of a member of the library to a component of the sample is detected by fluorescence polarization.

5. The method of Claim 2 in which the molecular probes are crosslinked to a photoactivatable functional group.

30 6. The method of Claim 5 in which the functional group is activated and a chemical bond is formed between a probe and a component in the sample.

7. The method of Claim 2 in which the probes are labeled with a scintillant and the components of the sample are radiolabeled.

8. The method of Claim 7 in which the interaction between a probe and a component of a biological sample results in the emission of light.

5 9. The method of Claim 1 or 4 in which the members of the library are biomolecules.

10. The method of Claim 9 in which the biomolecules in the library are glycoproteins, lipoproteins, proteins, 10 polypeptides, peptides, peptoids, amino acids, polysaccharides, oligosaccharides, saccharides, polynucleotides, oligonucleotides, nucleotides, nucleosides, DNA or a derivative, RNA or a derivative, lipids, glycolipids, lipopolysaccharides, organic molecules or 15 inorganic molecules.

11. The method of Claim 10 in which the biomolecules in the library are receptors, ligands, antibodies, antigens, epitopes, growth factors, cytokines, or chemokines.

20 12. The method of Claim 1 or 4 in which the sample is a patient sample or a clinical sample.

25 13. The method of Claim 1 or 4 in which the sample is a body fluid, urine, lymph, whole blood, sera, plasma, red blood cells, white blood cells, tissues, cells, cell extracts, products from in vitro transcription or translation, viruses, microorganisms, or extracts of pathogenic organisms.

30 14. The method of Claim 13 in which the molecular fingerprint generated for the sample using the library of molecular probes is compared to a molecular fingerprint generated for a negative control sample using the library of molecular probes.

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15. The method of Claim 14, in which the negative control sample is derived from a normal subject.

16. The method of Claim 13 in which the molecular
5 fingerprint generated for the sample using the library of molecular probes is compared to a molecular fingerprint generated for a positive control sample using the library of molecular probes.

10 17. The method of Claim 16 in which the positive control sample is derived from a diseased or affected subject.

18. The method of Claim 1 or 4 in which the molecular
15 components in the sample are glycoproteins, lipoproteins, proteins, polypeptides, peptides, peptoids, or amino acids.

19. The method of Claim 1 or 4 in which the molecular components in the sample are polynucleotides,
20 oligonucleotides, nucleotides, nucleosides, DNA or a derivative, or RNA or a derivative.

20. The method of Claim 1 or 4 in which the molecular components in the sample are polysaccharides,
25 oligosaccharides, or saccharides.

21. The method of Claim 1 or 4 in which the molecular components in the sample are lipids, glycolipids, lipopolysaccharides or lipoproteins.

30 22. The method of Claim 1 or 4 in which the molecular components in the sample are organic molecules or inorganic molecules.

35 23. The method of Claim 1 or 4 in which the molecular components in the sample are receptors, ligands, antibodies, antigens, epitopes, growth factors, cytokines or chemokines.

24. An assay for characterizing biological samples, comprising:

5 (a) contacting a biological sample with a library of fluorescently labeled probes; and
(b) detecting the binding interaction for each fluorescently labeled probe with the biological sample.

25. A method for distinguishing biological samples, comprising:

10 (a) contacting biological samples with identical libraries of fluorescently labeled probes;
(b) detecting the binding interaction for each probe with each biological sample; and
15 (c) identifying a pattern of binding interactions which differentiates the biological samples from each other.

26. A method for identifying biological targets of therapeutic and diagnostic importance and lead structures for drug development, comprising:

20 (a) contacting two identical libraries of probes with two biological samples, wherein the first biological sample is a control;
25 (b) detecting the binding interaction between each probe and a component of the biological samples using a homogeneous assay system; and
30 (c) identifying probe/biological component binding interactions that are characteristic of the second biological sample, wherein a component so identified is a biological target and the probe having affinity for the biological component is a lead structure for drug development.

35 27. An assay for characterizing a sample composed of a mixture of molecular components, said assay comprising:

(a) attaching members of a library of molecular probes to scaffolds having a site cleavable by a specific catalyst and a marker on each side of the site, to provide molecular probe/scaffold constructs;

5 (b) combining the molecular probe/scaffold constructs with the sample, wherein a molecular probe having affinity for a molecular component present in the sample binds to the sample component forming a complex which blocks the cleavable site of the scaffold;

10 (c) adding the specific catalyst to the mixture such that scaffolds that do not include a bound sample component are cleaved; and

15 (d) detecting the release of unbound molecular probes from the scaffold or the retention of complexes on the scaffold;

15 in which the binding pattern of members of the library with the sample provides a molecular fingerprint of the sample.

20 28. The assay of Claim 21 wherein the scaffold is comprised of DNA, peptides, peptoids, or any polymer.

25 29. The assay of Claim 21 in which a ligand having affinity for a receptor present in a biological sample binds with the receptor and blocks the scaffold-specific agent's access to the scaffold.

30 30. The assay of Claim 21 wherein the scaffold-specific binding agent is a drug, peptide, oligopeptide, polypeptide, protein, glycoprotein, lipoprotein, saccharide, oligosaccharide, polysaccharide, organic or inorganic molecule.

35 31. The assay of Claim 21 wherein the scaffold-specific binding agent does not bind to the region of scaffold where the interaction between a ligand and a receptor occurs.

32. The assay of Claim 21 wherein the scaffold includes a marker that is blocked when the binding protein is bound to the screening sequence such that access to the marker indicates the absence of the scaffold-specific binding agent 5 and the presence of the ligand/receptor interaction.

33. The assay according to Claim 21 wherein the ligands are attached to the scaffold on the same side of the site as a first marker, and the assay further comprises 10 immobilizing the scaffolds at the first marker and washing the mixture after the addition of the catalyst to remove the unimmobilized portions of cleaved scaffolds, such cleaved portions including a second marker, and wherein intact scaffolds are identified by the presence of the second 15 marker.

34. The assay of Claim 27 wherein the scaffold is comprised of double-stranded DNA and the marker is a biotinylated nucleotide.

20 35. An assay for distinguishing biological samples of the same type, comprising:
(a) attaching members of identical libraries of probes to scaffolds that have a cleavable site and 25 markers on both sides of the site;
(b) contacting biological samples with the identical libraries of probes and the identical specific catalyst;
(c) detecting the binding interaction for each 30 probe with each biological sample; and
(d) identifying a pattern of binding interactions which differentiates the biological samples from each other.

35 36. The assay of Claim 30 which further comprises utilizing a control biological sample and identifying probe/biological component binding interactions that are

characteristic of the non-control biological sample, wherein a component so identified as a biological target and the probe having affinity for the biological component is a lead structure for drug development.

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37. A method for synthesizing a library of labeled ligands comprising:

10 (a) immobilizing a fluorescent dye to a solid phase support via a cleavable linkage thereby forming a first product mixture;

15 (b) reacting said first product mixture with one or more compounds or mixtures of compounds at a temperature and for time sufficient to form a second product mixture; and

20 (c) cleaving the product mixture from the solid phase support to form a library of labeled ligands; wherein the fluorescent dye comprises two reactive moieties independently selected from the group consisting of halogens, thiols, alcohols, ethers, esters, aldehydes, ketones, carboxylic acids, nitros, amines, amides, silanes, and protected and unprotected derivatives thereof.

38. The method of Claim 37 in which the fluorescent dye 25 is a rhodamine derivative, cyanine derivative, fluorescein derivative, tryptophan derivative, coumarin derivative, naphthyl derivative, bipyridine derivative, tripyridine derivative, organometallic complexes.

30 39. The method of Claim 37 in which the solid phase support comprises a linker, where in the linker is a halogen, thiol, alcohol, ether, ester, aldehyde, ketone, carboxylic acid, nitro, amine, amide, silane, or protected and unprotected derivatives thereof.

40. A method for synthesizing a library of labeled ligands comprising:

(a) immobilizing compounds on a solid support via a cleavable linker;

5 (b) attaching a fluorescent dye to the immobilized compounds; and

(c) cleaving the molecule-fluorescent dye reaction product from the solid support to yield a library of fluorescent-labeled ligand.

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41. A method for characterizing the toxic properties of a test agent comprising:

(a) contacting two identical libraries of probes with two biological samples, wherein the first biological sample is a control and the second biological sample was exposed to the test agent;

(b) detecting the binding interactions between each probe and the biological samples; and

(c) identifying probe/sample binding interactions that are characteristic of the second biological sample.

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42. A method for determining the presence of a toxic agent in a biological sample which comprises:

25 (a) contacting two identical libraries of probes with two biological samples wherein the first biological sample is a positive control which had been exposed to the toxic agent;

30 (b) detecting the binding interactions between each probe and each biological samples; and

(c) identifying probe/toxic agent binding interactions in the second biological sample that are characteristic of the positive control sample.

35

43. The method of Claim 41 or 42 in which the binding interactions between the probes and biological samples are detected using a homogeneous assay system.

5 44. The method of Claim 43 in which the library of probes are fluorescently or chemiluminescently labeled.

45. A method for characterizing the protein constituents of a sample, comprising generating a molecular 10 fingerprint of the glycoproteins, lipoproteins, proteins, polypeptides, peptides, peptoids, or amino acids in the sample using the method of Claim 1 or 26.

46. A method for diagnosing a disease or condition in a 15 patient, comprising:

(a) generating a molecular fingerprint of a biological sample obtained from the patient using the method of Claim 45; and

20 (b) comparing the molecular fingerprint of the patient sample to the molecular fingerprint of positive or negative control sample.

25 47. A method for prognosing the efficacy of a drug or therapy in a patient, comprising:

(a) generating a molecular fingerprint of a biological sample obtained from the patient using the method of Claim 45; and

30 (b) comparing the molecular fingerprint of the patient sample to the molecular fingerprint of patients who (i) respond to the drug or therapy, and to those who do not respond, to the drug or therapy; or (ii) demonstrate 35 adverse reactions to the drug or therapy.

48. A method for monitoring the efficacy of a therapeutic treatment in a patient, comprising:

5 (a) generating a molecular fingerprint of a biological sample obtained from the patient before treatment and periodically thereafter using the method of Claim 45; and

10 (b) comparing the molecular fingerprints generated for the patient samples to the molecular fingerprint of a positive or negative control sample.

15 49. A method for characterizing the nucleotide constituents of a sample comprising generating a molecular fingerprint of the polynucleotides, oligonucleotides, nucleotides, nucleosides, DNA or a derivative, or RNA or a derivative in the sample using the method of Claim 1 or 26.

20 50. A method for diagnosing a disease or condition in a patient, comprising:

25 (a) generating a molecular fingerprint of a biological sample obtained from the patient using the method of Claim 49; and

30 (b) comparing the molecular fingerprint of the patient sample to the molecular fingerprint of a positive or negative control sample.

51. A method for prognosing the efficacy of a drug or therapy in a patient, comprising:

35 (a) generating a molecular fingerprint of a biological sample obtained from the patient using the method of Claim 49; and

5 (b) comparing the molecular fingerprint of the patient sample to the molecular fingerprint of patients who (i) respond to the drug or therapy, and to those who do not respond to the drug or therapy; or (ii) demonstrate adverse reactions to the drug or therapy.

52. A method for monitoring the efficacy of a therapeutic treatment in a patient, comprising:

10 (a) generating a molecular fingerprint of a biological sample obtained from the patient before treatment and periodically thereafter using the method of Claim 49; and

15 (b) comparing the molecular fingerprint generated for the patient samples to the molecular fingerprint of a positive or negative control sample.

53. A kit for characterizing the molecular components of a sample, comprising:

20 (a) a library of molecular probes; and

25 (b) a means for detecting binding of the members of the library to molecular components in the sample in a homogeneous or liquid phase.

54. The kit according to Claim 53 in which the library of probes are labeled with a fluor, and the detection means is a fluorescence polarization instrument.

30 55. The kit of Claim 54 which further comprises a reaction vessel means for each member of the library suitable for conducting a homogeneous assay.

35 56. The kit of Claim 54 which further comprises a scaffold to which the members of the library are releasably attached.

57. The kit of claim 54 in which the library of molecular probes are selected to bind with molecular components associated with diabetes, infectious disease, inflammatory disease, heart disease, neoplastic disorders, 5 autoimmune disease and central nervous system disorders.

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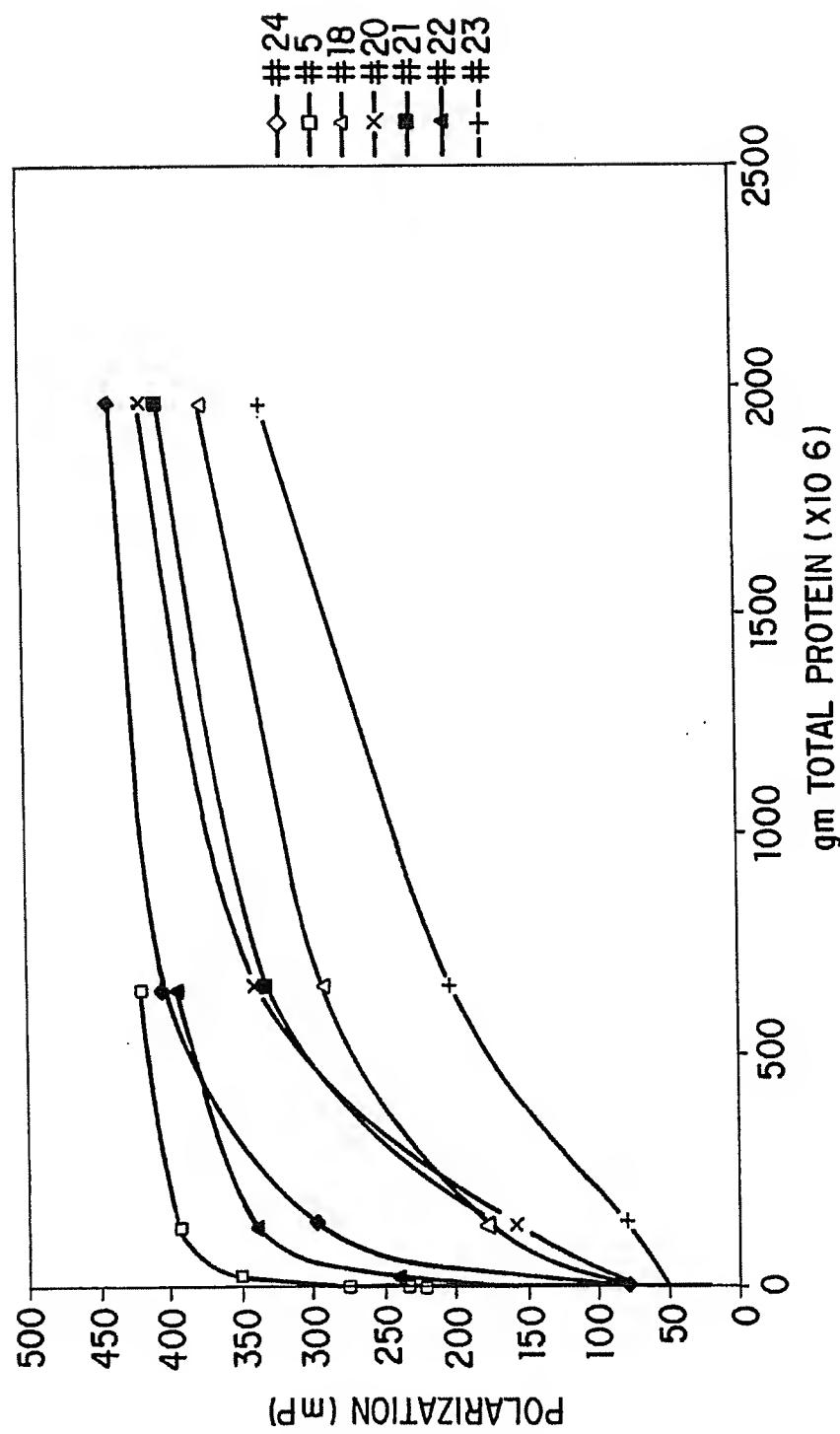


FIG. 1

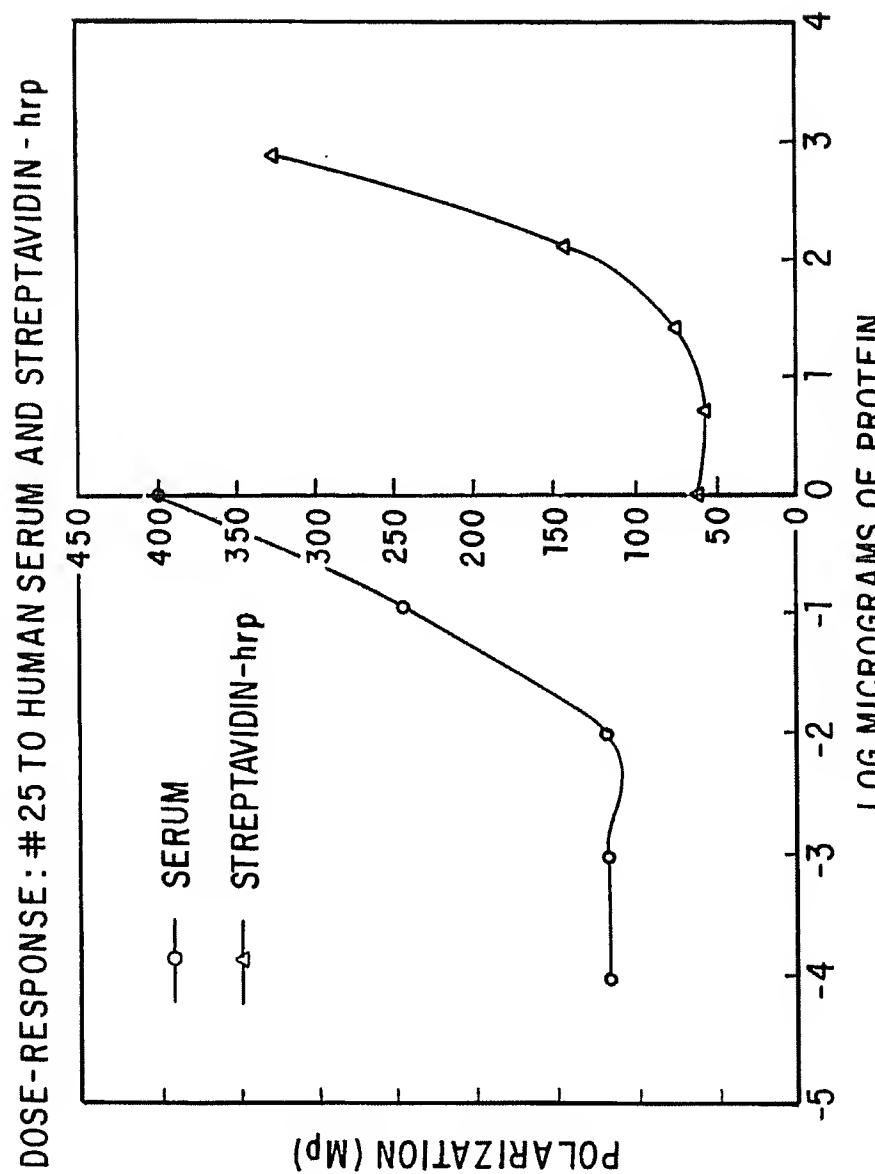


FIG. 2

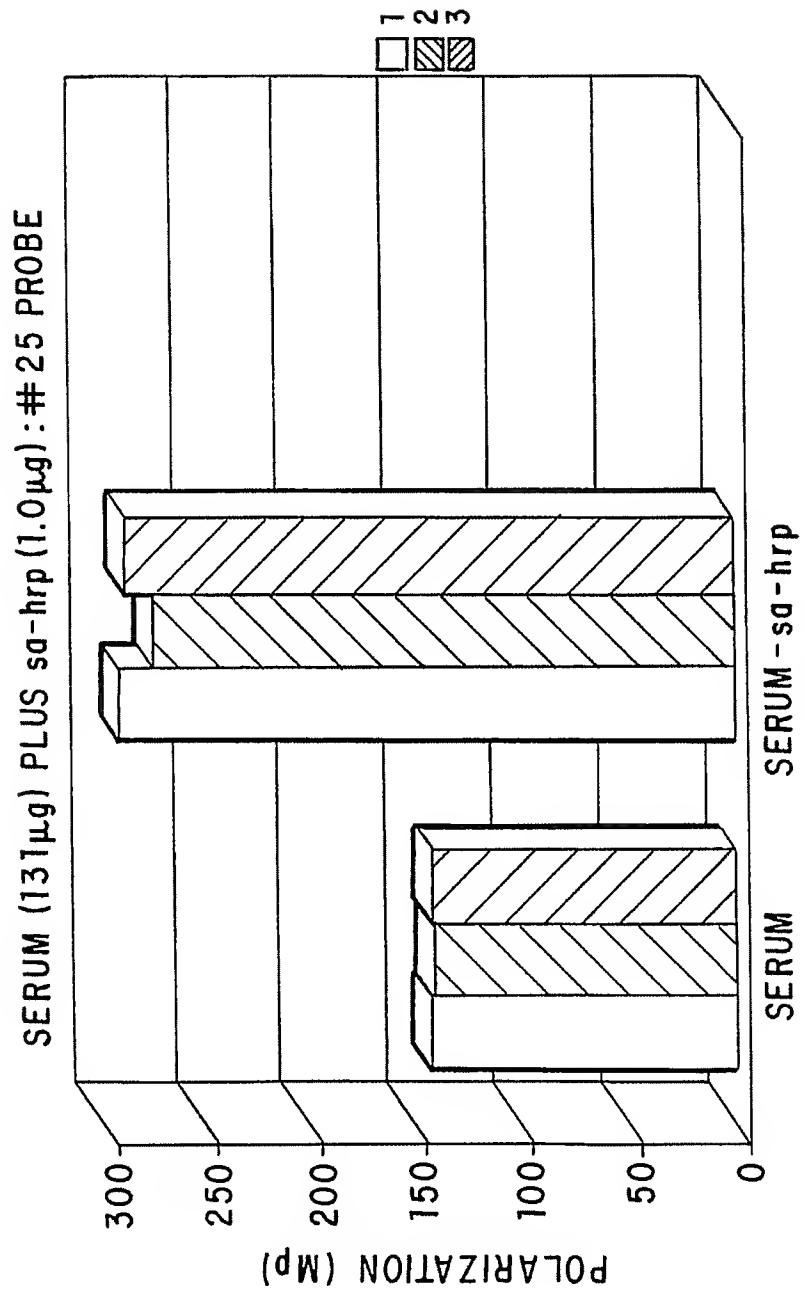


FIG. 3

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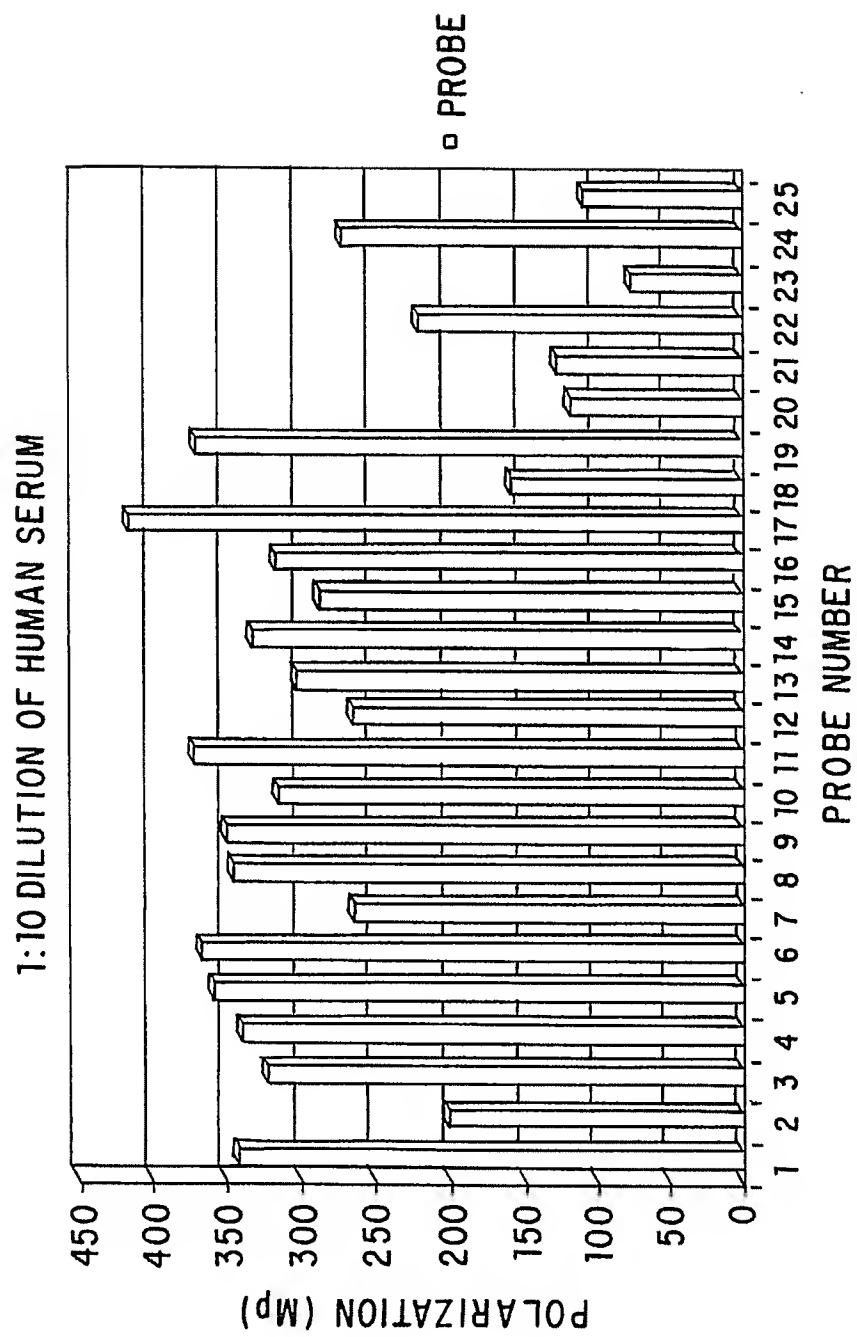


FIG. 4

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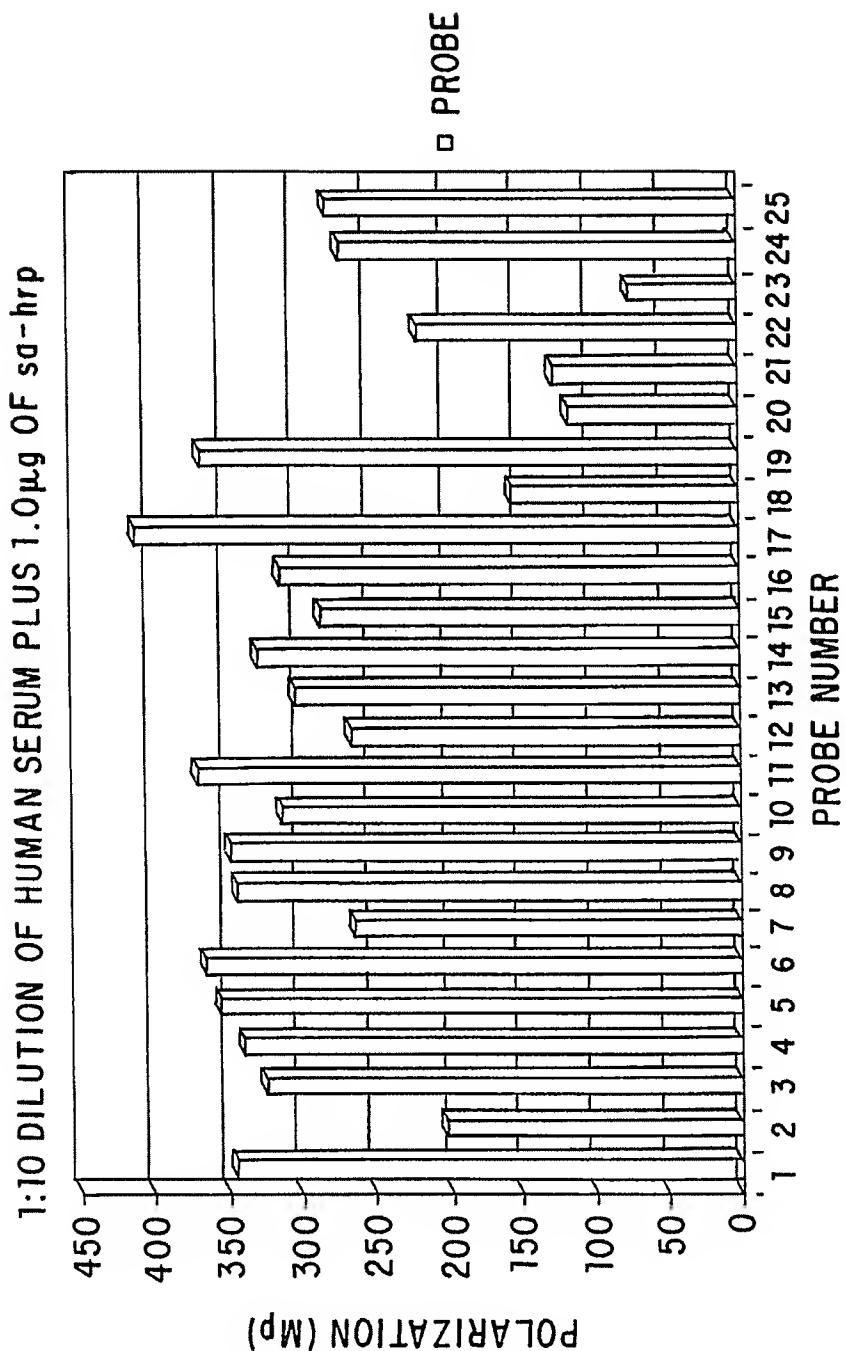


FIG. 5

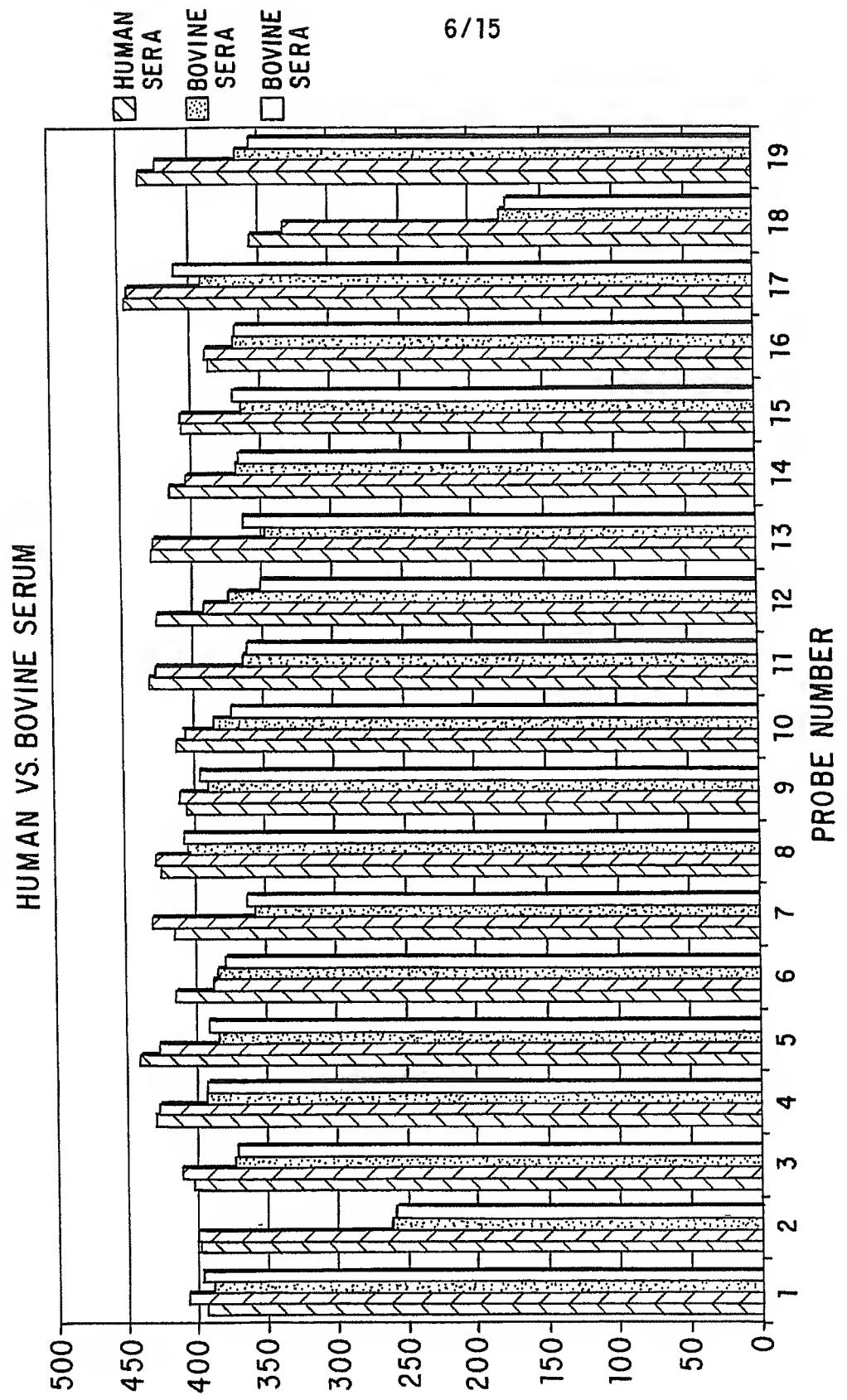


FIG. 6

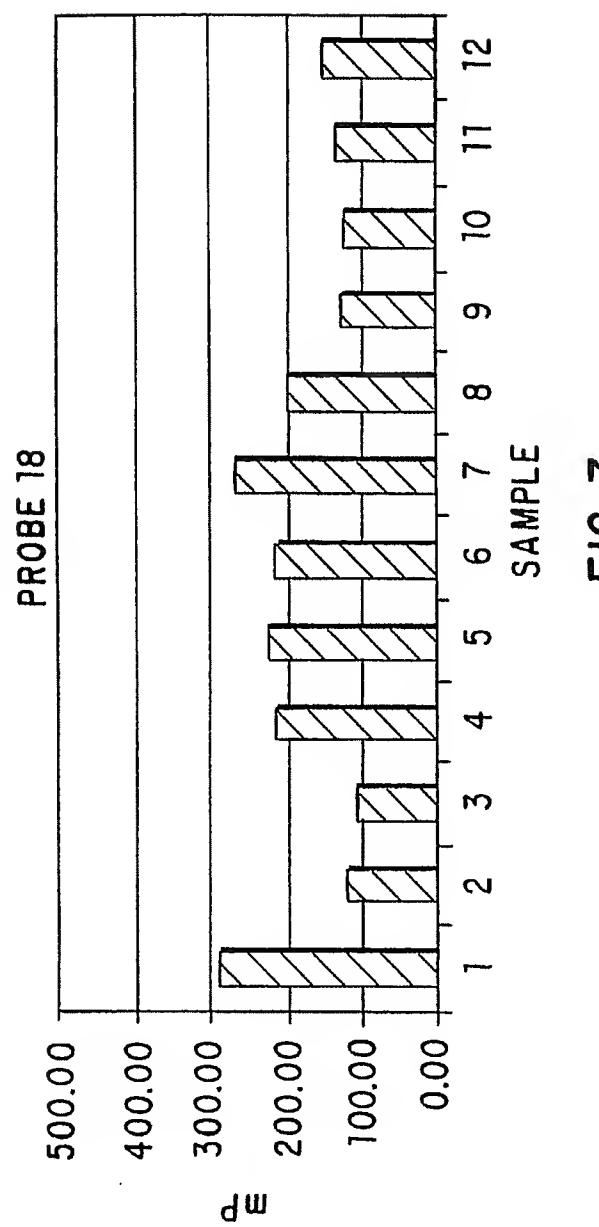


FIG. 7

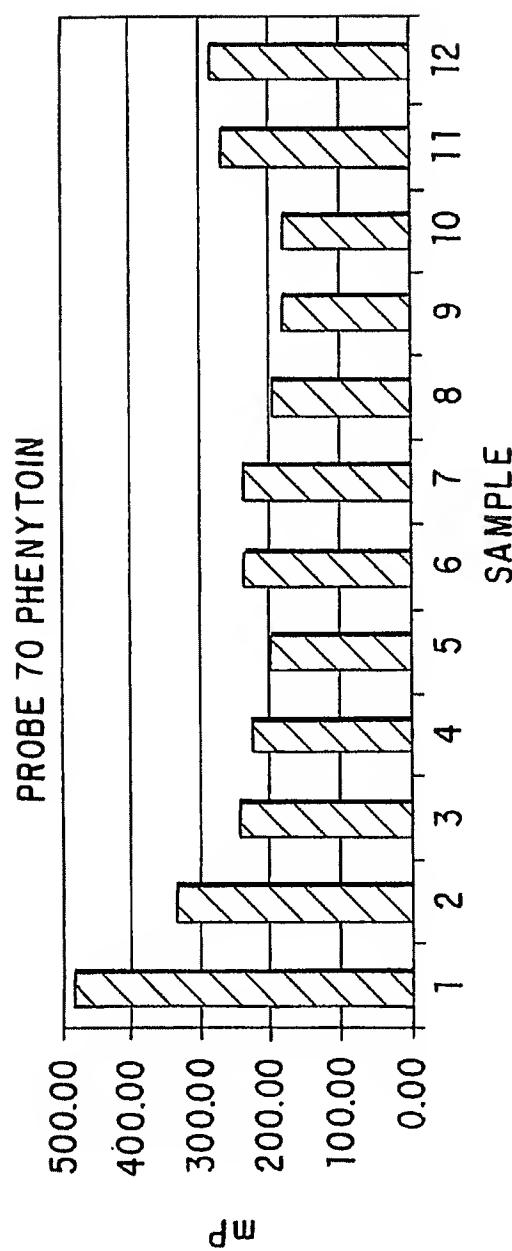


FIG. 8

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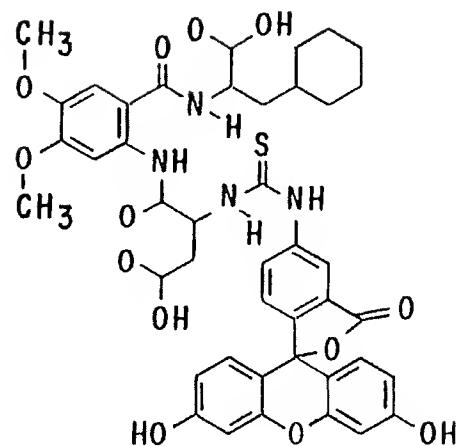


FIG 9A

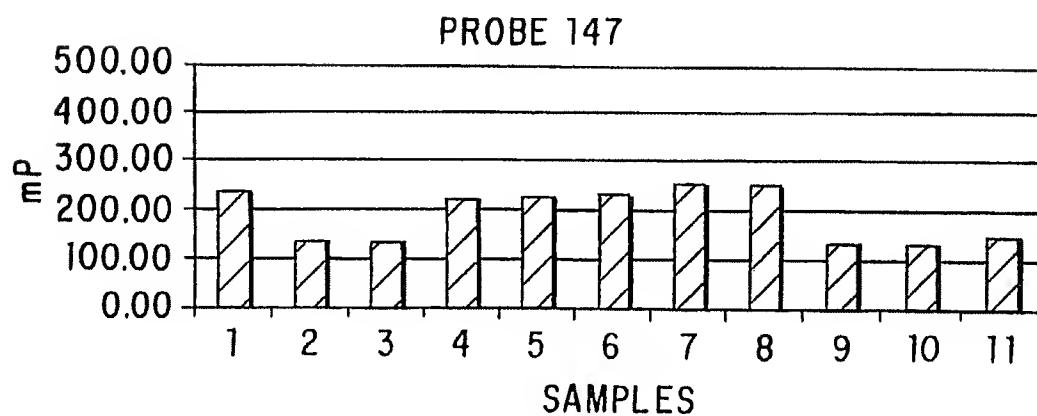


FIG. 9B

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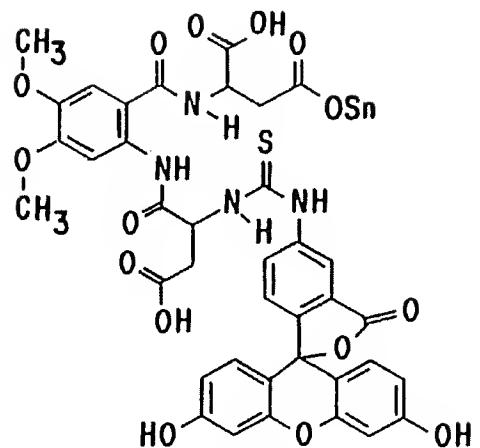


FIG. 10A

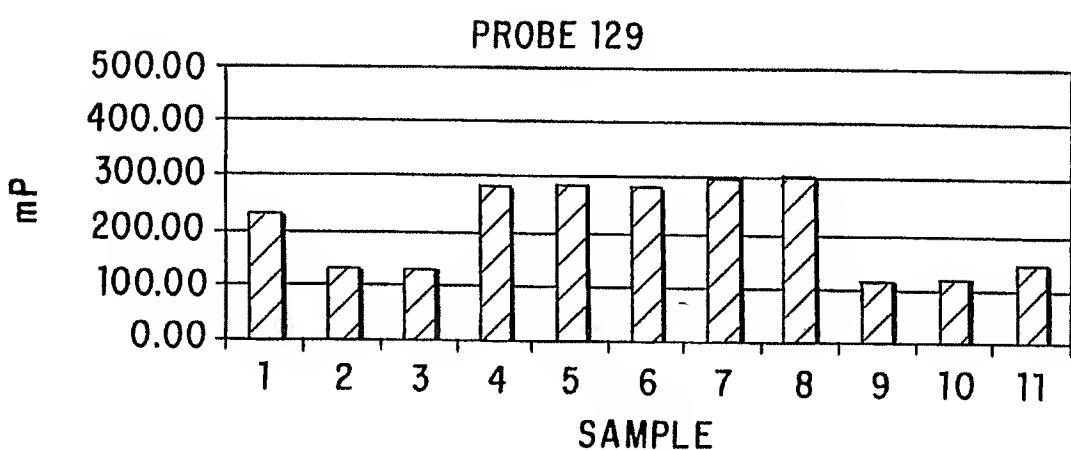


FIG. 10B

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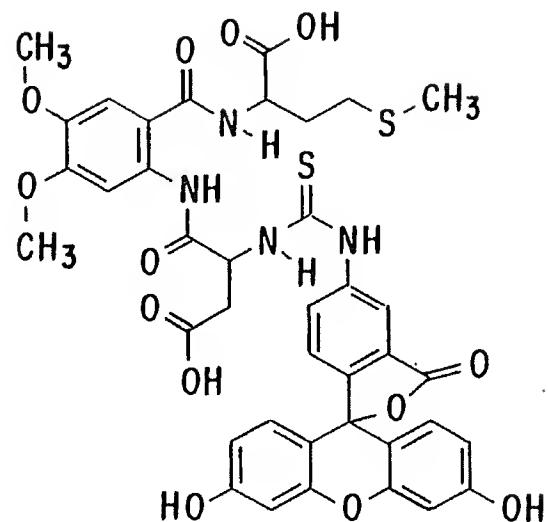


FIG. 11A

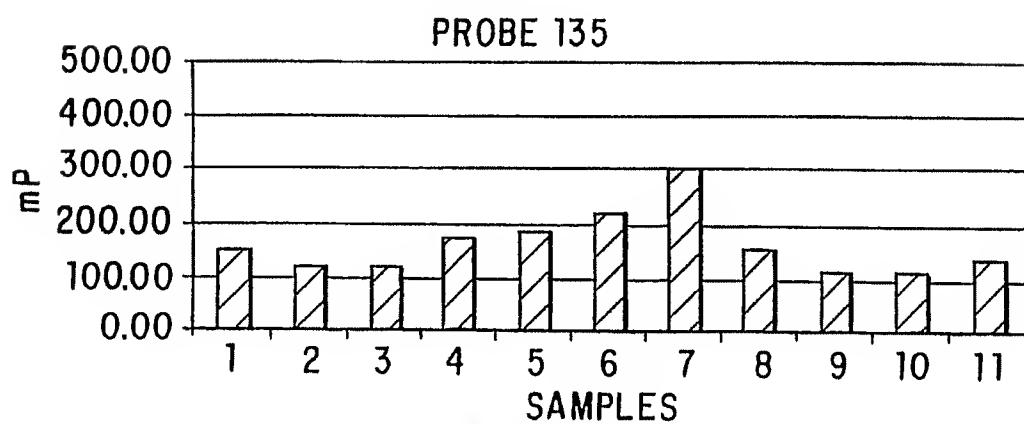
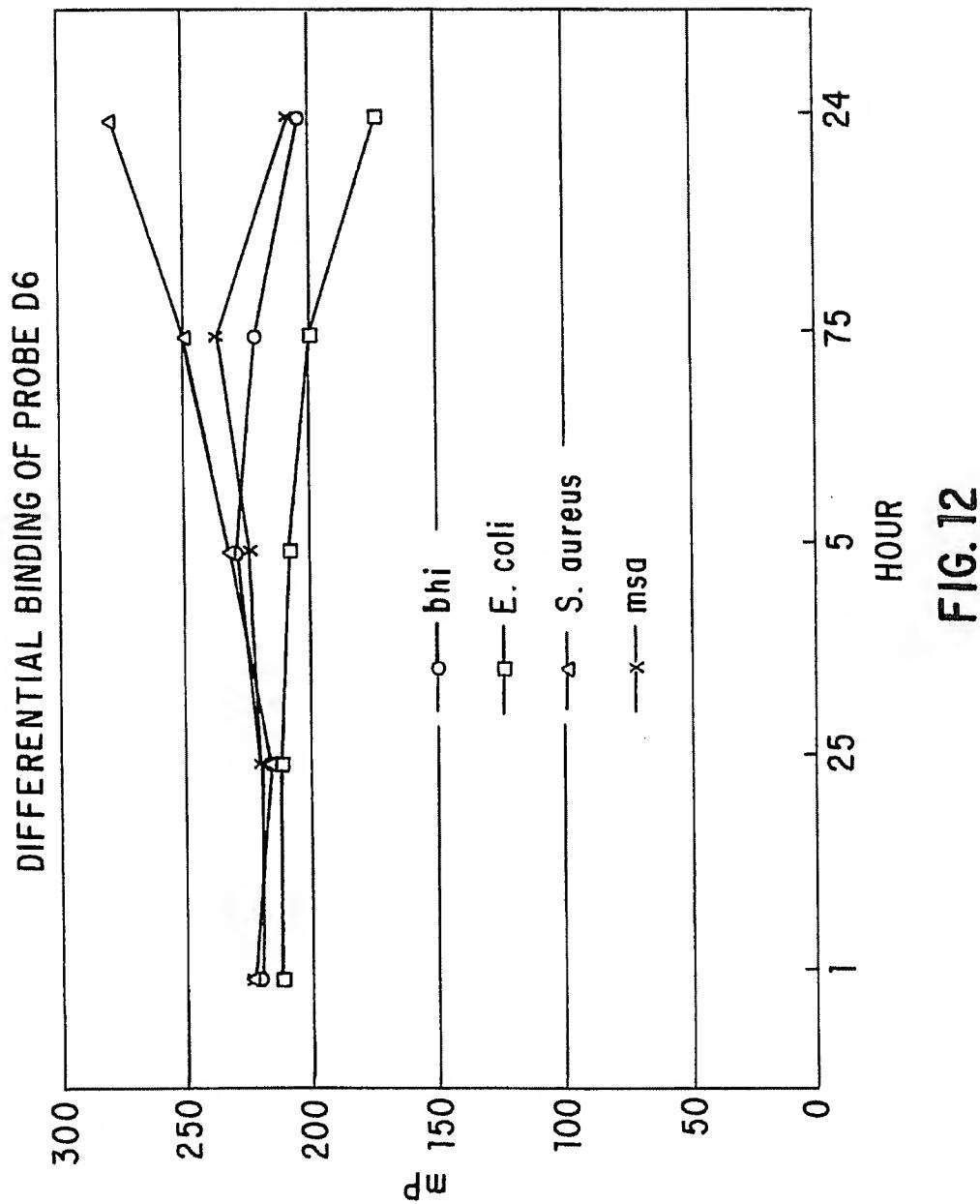


FIG. 11B

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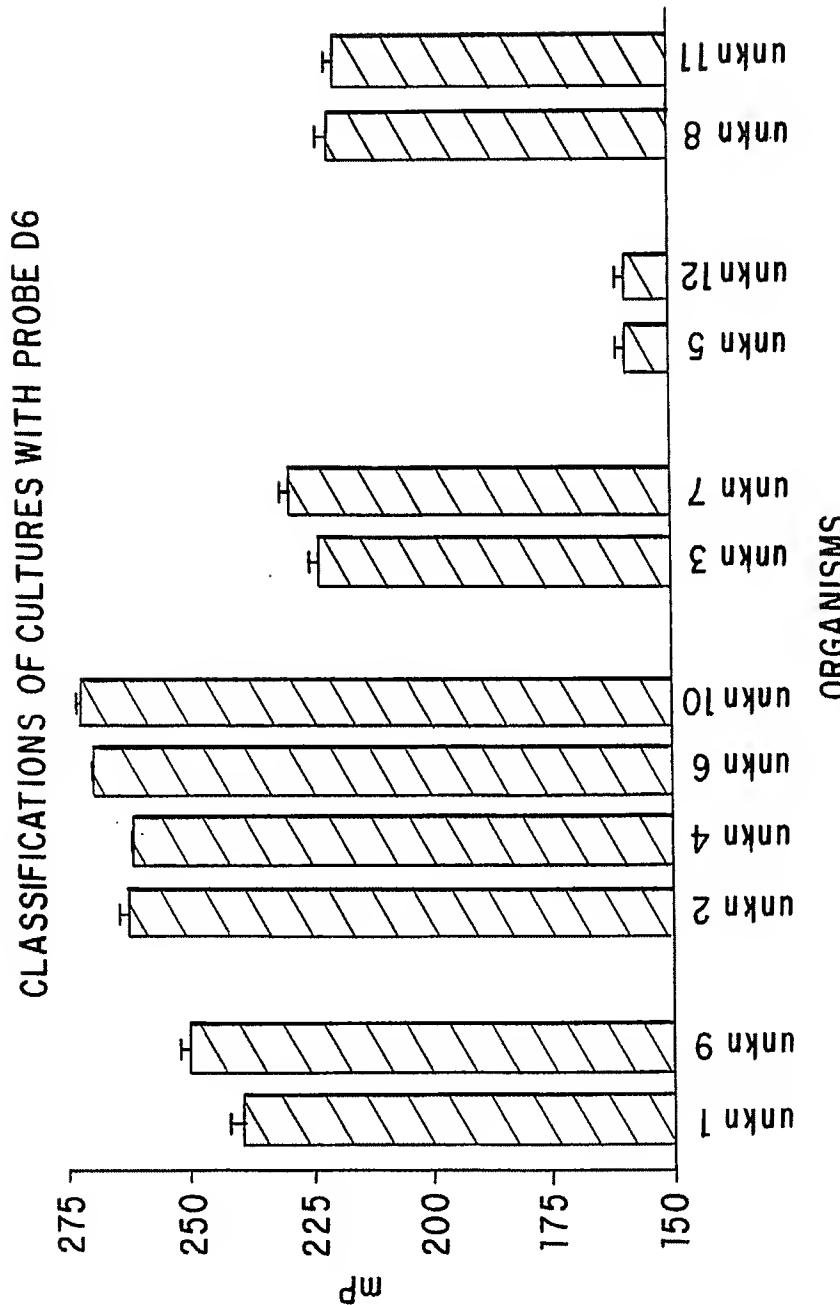
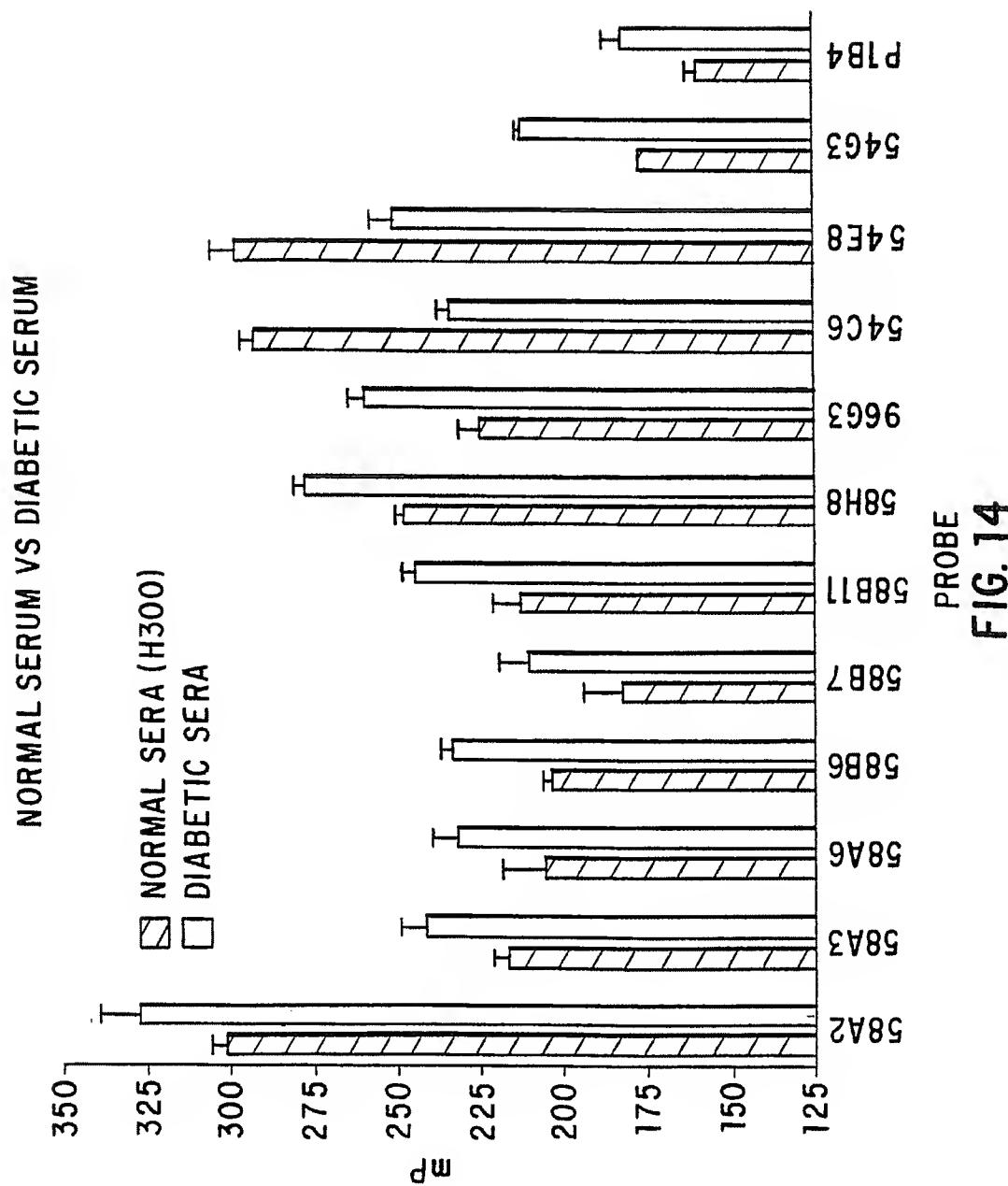


FIG. 13

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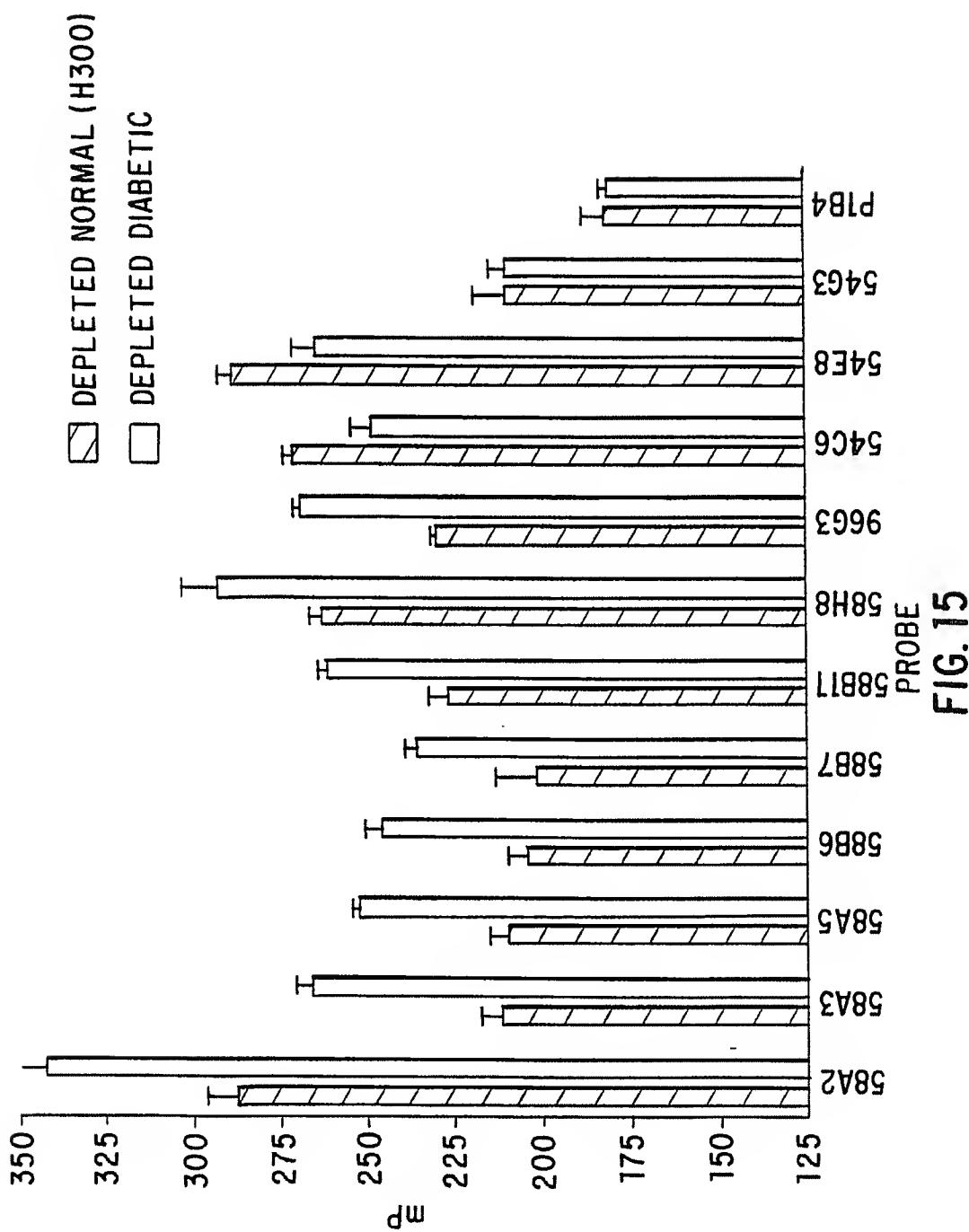


FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26894

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :435/4, 5, 6, 7.1, 7.21; 436/501, 503, 504, 164, 172, 808

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 5, 6, 7.1, 7.21; 436/501, 503, 504, 164, 172, 808

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

search terms: libraries, molecular probes, homogeneous assay, fingerprint, fluorescence polarization

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92/17784 A1 (TERRAPIN TECHNOLOGIES, INC.) 15 October 1992, see abstract, pages 5, line 1 to page 15, line 10.	1-24, 26, 41-46, 53-57
Y	US 4,568,649 A (BERTOGLIO-MATTE) 04 February 1986, see entire document.	1,2,7,8, 41, 42
X	US 5,541,070 A (KAUVAR) 30 July 1996, see entire document but particularly column 5, line 26 to column 26, line 63.	1-24, 26, 41-46, 53-57
X,P	US 5,741,653 A (KAUVAR et al) 21 April 1998, see entire document but particularly column 5, line 5 to column 15, line 52.	1-24, 26, 41-46, 53-57

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z"	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search Date of mailing of the international search report

09 MARCH 1999

02 APR 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

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Telephone No. (703) 308-3804

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26894

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIN et al. A Porous Silicon-Based Optical Interferometric Biosensor. Science. 31 October 1997, Volume 278, pages 840-843, see entire document.	1-24, 26, 41-46, 53-57

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26894

A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C12Q 1/00, 1/68, 1/70; G01N 33/53, 33/566, 33/567, 21/00, 21/76

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-24, 45, and 49, drawn to a method of characterizing samples using a molecular library.

Group II, claim 25, drawn to a method distinguish samples using identical plural libraries.

Group III, claim(s)26, 45, and 49, drawn to method of identifying biological targets (drug leads) using test and control libraries.

Group IV, claims 27-34, drawn to a method of characterizing samples using scaffold-attached molecular probes (with catalyst cleavable linkages).

Group V, claims 37-40, drawn to a method of synthesizing a library.

Group VI, claims 41-44, drawn to a method of characterizing properties of a toxic agent using finger print of claim 45.

Group VII, claim 46, drawn to a method of diagnosing a disease.

Group VIII, claim 47, drawn to a method of prognosing drug efficiency using finger print of claim 45.

Group IX, claim 48, drawn to a method of monitoring efficiency of treatment using finger print of claim 45.

Group X, claims 49-52, drawn to a method of characterizing nucleotide constituents.

Group XI, claims 53-57, drawn to a kit.

The inventions listed as Groups I-XI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The various methods recited in groups I-IV and VI-X have different objectives and use different method steps or are drawn to different use of a molecular library. Group V is drawn to method of making a library which is not the same library that is used in method of the above groups. Group XI is drawn to kit which may comprise a library different from the library made or used in the other groups. This is so because the chemical characteristics of the library is not specified and there is nothing in the claims that would indicate that a common library is made and employed in the various methods claimed.

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October 25, 2010

Via E-Mail (jwsuh@unhwa.net)

Mr. Jangwon Suh
Unhwa Corporation
4F Shinil Tower
68-22 Chungdam-dong, Gangnam-gu
Seoul, Korea 135-100

Re: U.S. Patent Application No.: 61/406,129
Title: PLANT CELL LINES AND METHOD OF ISOLATING THE SAME
Inventors: Eun-Kyong Lee et al.
Your Reference: *Please advise.*
Our Reference: 87374(307106)

Dear Mr. Seo:

The above-referenced patent application was filed on October 23, 2010 with the United States Patent and Trademark Office (USPTO). Attached is a copy of the document(s) as filed with and received from the USPTO. Our invoice will be sent to you soon.

Please note that U.S. non-provisional applications and foreign applications claiming the benefit of the filing date of this application must be filed on or before October 23, 2011. We will remind you of the October 23, 2011 deadline.

Please contact us if you have any questions, or if we can be of further assistance.

Best regards,

Kongsik Kim

KK/cm
Enclosures